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THE MORPHOGENETIC FUNCTIONS OF THE EPIDERMAL AND DERMAL COMPONENTS OF THE PAPILLA IN FEATHER REGENERATION[1]

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I. INTRODUCTION

THE present study is an outgrowth of earlier experiments (Lillie and Wang, 1941) on the analysis of feather morphogenesis. The problem arose from the fact that the papilla is of composite origin, consisting of a dermal core and a thin covering of follicular epidermis, the so-called "regeneration cells"[2] (Lillie and Juhn, 1938). Since the feather proper is formed exclusively by the epidermal cells, the question arises whether the dermal core of the papilla participates in morphogenesis in addition to its well-known nutritive function (Lillie, 1940). Lillie and Wang (1941) ascribed a fundamental morphogenetic function to the dermal component of the papilla, as a result of their experiments, viz., the determination of the symmetry and orientation of the feather by means of induction of bilateral organization in the ectoderm of the papilla, which was regarded as originally totipotent. But, as both derma and epidermis were involved in the experiments, the proof of this conclusion remained indirect.

Although it was found that breast and saddle papillae of the Brown Leghorn male or capon, when transplanted reciprocally between breast and saddle tracts, give rise to feathers specific to their respective donor tracts in both structure and pigmentation, this did not reveal whether the persistence of the donor tract specificity in the graft was due to its dermal or its epidermal constituent. These experiments, as well as others (feather germs transplanted under

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[2] The term "regeneration cells" is used throughout this paper as equivalent to the epidermal component of the papilla. These cells give rise to the entire epidermal coat of the papilla from which the collar of a regenerating feather is derived; however, owing to the fact that they represent a thickened portion of the follicular epidermis adjacent to the papilla, their existence does not terminate sharply at the boundary of the papilla but continues into the epidermis of the bottom of the follicle, i.e., surrounding the base of the papilla (see Lillie and Wang, 1941, Fig. 3). The latter portion of regeneration cells remains with the follicle when the papilla is removed.

the skin and chimaera feathers from combined sagittal half-papillae of different tracts), prove that an intact papilla or even part of it, but with both the epidermal and dermal components, regenerates true to its tract of origin, no matter where it may have been transplanted autoplastically. It remains to be decided, however, whether tract specificity is determined by either the dermal or the epidermal component to the exclusion of the other, or possibly by both jointly. The fact that the epidermal cells actually build the complete feather does not necessarily exclude the possibility of induction of tract specific characters by the dermal component. The question cannot be settled so long as the transplanted papillae are tested with both the epidermal and the dermal components coming from the same tract.

In order to delimit the morphogenetic roles of the two components of the papilla, a technique was devised by which the components of breast and saddle papillae could be separated and recombined. This was achieved by destroying the epidermal coat of a papilla and transplanting the denuded dermal core autoplastically into a follicle of the reciprocal tract, the papilla of which had been previously completely removed. Local epidermis of the host tract grew over the transplant, and a composite papilla was thus obtained, with the dermal portion coming from one tract and the epidermal portion from the other. The resulting feather was studied to ascertain the extent to which the contributing tracts had determined its characteristics.

Criteria used for differentiating breast and saddle feathers in Brown Leghorn capons are: shape, form, total length, proportion of fluff, and the structure of the barbs. In addition, growth rate and pigmentation, which are specific for the two tracts, were also used as diagnostic features. The use of pigmentation in this connection was limited, however, by the lack of definite knowledge of the site of the potential melanophores in the definitive papilla. (Willier and Rawles, 1940, p. 195), as well as of the specificity of the melanophores responsible for the contrasting color of the feathers of the two tracts (Ris, 1941, p. 60).

The technique was also used to check the earlier finding (Lillie and Wang, 1941) regarding the seat of determination of symmetry of regenerating feathers. Rotation of denuded dermal papillae prior to implantation into empty follicles made it possible to analyze the contribution of the separate components in the determination of feather orientation. Finally, an attempt was made to find the extent of the feather-forming capacity of the follicular epidermis by implanting denuded dermal papillae into empty and normal follicles at different levels of the follicular wall.

The purpose of this investigation, therefore, was (1) to delimit the morphogenetic functions of the epidermal and dermal components of the definitive papilla by (*a*) ascertaining the seat of tract specificity and (*b*) reinvestigating the seat of determination of symmetry in the regenerating feather and (2) to determine the extent to which general follicular epidermis, remaining after the removal of a papilla, is capable of feather regeneration.

II. MATERIALS AND METHODS

The experiments were performed on Brown Leghorn capons (except in three cases, see p. 341). Male chicks obtained from the Illinois Hatchery were castrated at the age of 4 weeks. The young capons were raised together and were kept on a well-balanced diet. Operations began when they were 4 months old and were

continued until they were a little over 1 year of age.

The following description of technique refers to the basic experiment; details pertaining to other types of operation will be given later. In searching for an agent for the destruction of the epidermal component of the papilla, alcohol, hot Ringer's solution, and weak solutions of alkali and inorganic acids were found to be unsatisfactory. Finally, salicylic acid dissolved in Collodion Flexible (Merck) proved successful. The ether and alcohol contained in this collodion evaporated rapidly on exposure to air, so that the viscous fluid promptly formed a film, i.e., a collodion coat, which could be readily stripped off. Immersion of isolated papillae for from 1 to 3 minutes in 25 per cent salicylic acid gave consistent results with a safe margin. The final formula of the mixture was fixed at 25 gm. of salicylic acid and 5 gm. of camphor (added as a germicide), dissolved in 100 cc. of the collodion. The standard duration of immersion was 1 minute, although a slightly longer exposure did not appreciably alter the results.

The most favorable sites for operation are the anterior breast and the extreme posterior saddle. (Hereafter breast and saddle will be designated as "B" and "S," respectively.) Feathers from these regions were plucked prior to each experiment, so that the regenerating feathers would be of the same regeneration age; usually, active B papillae were used after 14–35 days and S papillae after 14–45 days. For anesthesia, Nembutal (Pentobarbital Sodium, Abbott) was injected intraperitoneally (1 cc. per 5 pounds of body weight). All instruments were thoroughly cleaned, dried, and dipped in alcohol just before use. Solutions, cotton, and glassware were sterile.

For easier identification of operated follicles horizontal rows of adjacent follicles, not more than ten in a row, were chosen. Operation proceeded from the most anterior one posteriorly. (See Lillie and Wang, 1941, Figs. 2, 3, 11, and 12, for a general orientation.) After sterilizing the skin with alcohol, the feather was plucked, and a slit about two-thirds of the depth of the follicle was made with scissors. The bottom of the follicle was lifted up with two pairs of pointed steel forceps under the binocular microscope; the papilla was brought into focus and then extirpated. During this process the papilla was held and lifted by a little collar of subdermal tissue left attached to it. Papillae of both B and S tracts, if removed in one operation, were placed in separate, labeled dishes containing Ringer's solution. From the latter the papillae were removed, one at a time, to a piece of filter paper to remove excess liquid, and then were immediately transferred to a glass slide, apical ends up. A drop of the salicylic acid mixture, not over 6 mm. in diameter, was then placed over the papilla. After 1 minute the papilla was removed from the film by its base and was returned to Ringer's solution. The excess follicular wall attached to the base of the papilla was trimmed so as to give a fresh base free from chemical contamination. Care was taken not to injure the basal portion of the dermal component of the papilla during trimming. It was then transferred once more to fresh Ringer's solution and left there for about 30 minutes. The treated papilla was now ready to be implanted into an empty follicle of the reciprocal tract, i.e., either $B \rightarrow S$ or $S \rightarrow B$. Each denuded papilla was held lightly between the points of forceps while it was pushed toward the bottom of the recipient follicle, the incised wall of which was finally pulled together to heal. To insure successful incorporation of the

graft, the recipient follicle must not be left empty for more than 2 hours.

The arrangement of the operated follicles in rows removed the necessity of marking individual follicles. Adjacent feathers clipped immediately after the operation served as markers. As soon as the follicles were healed, the skin about the first and last in each row was tattooed with India ink. The bird was examined at intervals; and after the feathers had completely regenerated, they were plucked and studied individually.

The characteristics which constitute the respective tract specificities of B and S are given in Table 1. All experimental feathers were classified on the basis of these characters only.

TABLE 1

TRACT SPECIFICITIES OF BREAST AND SADDLE FEATHERS IN BROWN LEGHORN CAPON

FEATURES		TRACTS	
		Breast	Saddle
Form	Shape	Rounded apex with broad vane	Acuminate apex with slender vane
	Average length	10 cm.	15 cm.
Structure	Vane	A uniformly interlocked vane formed by barbs carrying barbules along their entire length	An incoherent vane formed by barbs carrying barbules only in basal portions to form a "web," leaving margins of vane "lacy"
	Fluff	Structurally similar in both tracts	
		About one-half of total length	About one-third of total length
Pigmentation	Vane	Solidly black throughout	Golden brown except near the base, where a spot of black is usually present
	Fluff	Grayish in both tracts	
Mean growth rate....		2.3 mm. per day	1.8 mm per day

III. EXPERIMENTAL

A. EPIDERMAL ORIGIN OF TRACT SPECIFICITY

1. *The basic experiment.*—An average of from 3 to 4 weeks elapsed before feathers emerged from the operated follicles. From the very first their pigmentation was characteristic: feathers resulting from S → B were black, while those from B → S had a golden-brown color. This unmistakable acquisition of host tract pigmentation by feathers regenerating from denuded papillae was consistent throughout the experiments. Its significance, as contrasted with the invariable retention of donor tract pigment from untreated (intact) papilla-transplants between the two tracts, will be treated fully in the section on discussion. As the feathers grew longer, they revealed the typical structural characteristics of the host tract in spite of the fact that most of them were morphologically defective. When they were about 4 mm. long, those with defects limited to the apical portion showed a vane characteristic of the host tract. Those defective over their full length, i.e., tuft feathers, formed barbs identifiable as of host tract character despite the absence of a rhachis. Later, when the fluff began to form, the general form and shape of the feathers were well established except for the tuft feathers. In each case these characters were found to be typical of the host tract: rounded tip and broad vane for those from S → B, and an acuminate tip and narrow vane with a "lacy" margin for those resulting from B → S. Microscopic study showed that in the tuft feathers grown on B the barbs possessed barbules throughout their length, whereas in those grown on S the apical portion was free from barbules, corresponding to the "lacy" margin of normal S feathers. This portion of

the tuft barbs, however, varied in length according to the total length of the barbs themselves. Thus, even in the absence of a rhachis and a vane, one of the most characteristic differences of the B and S tracts was readily established. All other gross features—length, shape, fluff proportion, and pigmentation—conformed precisely to those of the host tract.

grown next to each of these was similarly measured. The results were conclusive: from the time of their emergence from the follicle all five feathers grew at exactly the same rate as their controls. Regardless of the origin of the dermal papilla, the growth rate was thus typical of the tract in which the feather was growing.

TABLE 2

RESULTS OF THE BASIC EXPERIMENT*

Type of Transplantation	Expt. No. (Ser.)	No. of Papillae Treated	Resulting Feathers					
			Approx. Normal	Missing Apex	Half-Vane	Apical Tuft	Tuft	Failed
S→B	34†	8	5	0	0	3	0	0
	38†	9	2	0	0	0	0	7
	47†	7	2	2	0	0	0	3
	125†	6	1	0	0	2	2	1
	107	6	2	1	1	1	0	1
	129	7	4	0	2	0	0	1
	131	7	0	0	0	2	1	4
Total		50	16	3	3	8	3	17
Percentage		100	32.6	6.0	6.0	16.0	6.0	34.0
B→S	34†	8	2	3	0	0	0	3
	38†	9	3	0	0	3	0	3
	47†	7	1	1	1	0	0	4
	125†	6	1	0	0	2	0	3
	33	6	0	0	1	1	1	3
	45	10	0	0	0	1	2	7
	127	8	0	0	2	1	3	2
Total		54	7	4	4	8	6	25
Percentage		100	13.0	7.5	7.5	15.0	11.0	46.0

* All resulting feathers listed in the table possess the specific characters of the host tract; no specific characters of the donor tract appear.

† Reciprocal transplantation.

In order to ascertain whether the growth rate of the composite feathers conforms to the dermal or epidermal component, three second-generation feathers growing on B (Expt. 129, No. 3; Expt. 107, No. 2; Expt. 47, No. 5) and two growing on S (Expt. 34, No. 2; Expt. 38, No. 1) were measured from the time their predecessors were plucked until they were completely regenerated (see Table 2). For comparison, one control feather

Normal B and S feathers take about 8–10 and 10–12 days, respectively, to emerge after plucking. But in the case of feathers regenerating from denuded papillae this lag was increased to as much as 4 weeks following implantation. The delay was, however, considerably lessened in the later generations, a fact which indicates a progressive disappearance of conditions unfavorable to regeneration introduced by the grafting process. For instance, of the five second-gen-

eration feathers used above for measurement of growth rate, none emerged within 17 days. Two B feathers emerged after 17 days, the other after 18 days; the two S feathers emerged after 20 days. The amount of delay that persisted in the second generation may be due to a prolonged subnormal physiological condition of the grafted papillae (cf. Lillie and Wang, 1943). These same feathers were examined again in their fourth generation. It was found that all three B feathers emerged only 2 days, the S feathers 6 days, after their controls.

The results of ten experiments (four reciprocal and six one-way transplantations) are summarized in Table 2. A total of sixty-two feathers was obtained from one hundred and four denuded papillae. These feathers were classified arbitrarily, according to the degree of defect, into five classes: (1) approximately normal, ranging from normal to apical defect not exceeding 1 cm.; (2) missing apex extending to any length of the vane; (3) distinctly half-vaned; (4) vaneless or with apical tuft; and (5) tuft (rhachisless) feathers (see Pls. I and II). It may be noted in passing that the apparent differences between the two tracts in regard to percentages of successful incorporations and of production of normal feathers are without statistical significance.

The data indicate that feathers from denuded papillae never manifest the characteristics of the tract of origin but invariably those of the tract in which they have grown. Nor was a mixture of donor and host features ever found. The tract specificity must therefore be attributed to the epidermal, i.e., host, component of the regenerating papilla. This is further borne out by the demonstration that the new epidermal coat of the graft is of host origin.

Direct evidence of the fact that the epidermis of the treated papilla is destroyed and that the denuded graft subsequently acquires a new epidermal coat from the host follicle can be obtained by tracing the histological changes of the graft from the time of its implantation. Three series of transplants to B follicles, involving a total of twenty-one denuded S papillae, were made. Fourteen were recovered at 2, $4\frac{1}{2}$, 5, 6, and 7 days by dissecting out the entire follicles. These were fixed in Bouin's fluid, cut at 8 μ longitudinally and stained with Heidenhain's Iron Hematoxylin. Representative stages from the 2-, $4\frac{1}{2}$-, and 7-day series are shown in Plate V, Figures 20, 21, and 22.

General observation, as well as histological study, of the recovered grafts revealed slight hemorrhage at the bottom of the wounded follicles, apparently dating back to the time of implantation. The resulting blood clot closed the cavity of the follicle distal to the apical end of the graft. No blood clot was found between the follicular wall and the graft, probably because of the pressure exerted by the follicular muscles. This pressure insures firm contact of the graft with all sides of the follicle except at its apex, a condition believed to favor successful incorporation.

Vascularization of the graft was established about 2 days after implantation. Sections recovered by that time gave evidence that the dermal papilla had been denuded and that epidermis of the host follicle was migrating over the implant. The basal portion of the graft was covered by epithelial cells, while its apical portion was still naked (see Pl. V, Fig. 20). That the direction of their movement was from base to apex was indicated by a "spearhead" of a single layer of epithelial cells in front of the much thicker sheet that had made a sharp turn from the bottom of the follicle up along

the surface of the graft. The activity of this invading epidermis appeared to have been stimulated by the presence of the graft, as indicated by the extensive thickening of the epidermal layer along the region of contact with the graft. Numerous mitotic figures were found around the entire cut edge of the follicle in direct contact with the graft in the region where the supplanting sheet of epidermis was thickest.

This epidermal replacement was advanced about half the length of the graft at $4\frac{1}{2}$ days (Pl. V, Fig. 21) and was completed by the end of the sixth or seventh day, when the entire graft became covered by new epidermis (Pl. V, Fig. 22). During this same period keratinization of the outer layers of the epidermal sheet occurred in the same manner as is observed in the outer margin of the collar in normal regenerating feathers. Meanwhile, the follicular cavity became filled with darkly stained keratin fibers. An initial differentiation of the epidermal cells into two layers appeared: inner cuboidal (Malpighian) cells with darkly stained nuclei and outer, more flattened cells destined to form keratin. The former layer normally gives rise to the differentiating zone of the future collar, although none of the series studied had as yet reached that stage, corresponding to the delay of emergence of feathers from grafts.

Up to 4 days after implantation the entire apical portion of the graft, in contrast to its basal portion, appeared bare and definitely necrotic. Farthest away from the source of vascularization and most exposed, the apical end obviously sustained the most severe damage. In a majority of cases it underwent degeneration and was eventually sloughed off.

A special effort was made to determine the fate of the destroyed epidermis. The sections revealed that a large part of the dead epidermis had been stripped away from the graft, forming a part of the debris usually attached to its apex. Occasionally some remnants were seen to be loosely connected with the periphery of the graft, appearing as poorly stained, structureless fragments, often sandwiched in between the base of the graft and the incoming epidermis of the host follicle. Other cells of the injured epidermal coat had undoubtedly undergone necrosis; the apical end of the graft, especially in younger stages (2–$4\frac{1}{2}$ days) was crowded with cellular aggregates composed of erythrocytes and lymphatic elements. Not infrequently large macrophages were also found here. These myeloid elements were probably of host origin, since, in most cases where necrosis was extensive, host blood capillaries could be traced from the affected area to the base of the graft. It was largely by these two means that the dead epidermis was finally disposed of. A third remnant of the original epidermis was sometimes seen near the lower portion of the graft in the form of islands of faintly stained cells caught in the middle of the dermis at the boundary between the graft and the host subdermal tissues.

Careful examination of sections in every series gave no indication of any infiltration of host subdermal tissue into the graft. Since the graft, composed of lightly staining dermal tissue, can be readily distinguished from the more darkly staining host tissue, any invasion by the subdermis would have been readily detected. In all sections up to 7 days after implantation the boundary between the graft and the host follicle remained distinct, although they were completely fused. Hence, the histological evidence indicated that the newly established composite papilla consisted of the dermal component of the donor tract and the epidermal component of the host tract.

An important point that required examination concerned the possibility that the denuded graft might have stimulated the regeneration of a whole new papilla of host origin. Although the histological studies showed nothing of this kind, two experiments were devised to test the possibility more directly. In the first experiment two pulps from freshly plucked, actively regenerating B feathers were isolated (freed from barbs) and cut transversely into ten pieces of about 2 mm. each. Ten B follicles of the same bird were deprived of their papillae, and a piece of the isolated pulp tissue was implanted into each of them. After 5 weeks nothing could be detected externally; the follicles appeared as empty as "sterile" ones (i.e., those that normally remain inactive as a result of the complete removal of their papillae). Upon dissection it was found that each had healed without leaving any trace of the grafted pulp; the latter presumably had dried up. The experiment was repeated twice with consistently negative results.

In the second experiment subdermal tissue, freshly dissected out of B follicles, was cut into ten pieces of the size of papillae, and each of these was autoplastically implanted into a freshly prepared, empty B follicle. The operated follicles produced no feathers. When, at the end of 5 weeks, the follicles were dissected, no grafts were found; obviously, they had undergone the same fate as the pulp tissue. There was no sign of regeneration when the experiment was repeated.

The negative results of these two experiments strongly suggest that a follicle, once deprived of its papilla, cannot replace the latter. This is not surprising, in view of the fact that both the dermal core of the definitive papilla and its underlying subdermal tissue have reached irrevocable differentiation, providing no reserve from which the regeneration of a new dermal papilla might draw. In this connection one recalls that structural defects in experimental feathers persisted throughout successive generations (Lillie and Wang; 1941, 1943), a condition apparently due to the inability of a papilla to repair its own losses. The fact that an empty follicle will remain inactive except in the presence of a grafted papilla, intact or denuded, demonstrates the existence of some specific affinity between the dermal core of a papilla and the follicular epidermis. The dermal component is thus indispensable for normal feather regeneration and is irreplaceable even though it is not specific between follicles of the same tract or between those of different (B and S) tracts.

In the light of the experimental evidence related above, one is led to the conclusion that all morphological distinctions (apart from symmetry) which, when taken together, constitute what we call "tract specificity" of a feather are determined exclusively by the epidermal component of the papilla, independent of the origin of the dermal component. The latter, being not tract specific, can consequently be interchanged between the tracts without affecting the tract specificity of the resulting feather.

The validity of this conclusion was further tested by transplanting an established composite papilla back to its original tract. In one experiment seven denuded S papillae were transplanted to B follicles and 3 days later implanted back to empty S follicles. Four of these failed; the remaining three gave rise to defective S feathers. The results indicated that the papillae involved in the double transfer had not been allowed sufficient time in the B tract to acquire a B epidermal coat before their return to the S tract. In a second experiment an equal number of S papillae were returned to S after having resided in B follicles for 8 days. These,

in sharp contrast to the results of the former group, yielded two definite, though defective, B feathers after a delay of 4 weeks, while the rest failed. This demonstrated the survival of the new epidermal component of B origin and its capacity to regenerate true to its tract regardless of the site of growth. In both experiments the unusually high percentage of failure possibly reflected the increased trauma involved in transplanting a treated papilla twice within a relatively short period.

2. *Other experiments.*—In order to further substantiate the significance of the basic experiment, several other experiments using slightly modified techniques were carried out. Some of them which involved local treatment of papillae—i.e., the *in situ* and "hot-needle" experiments—required the previous establishment of normal B and S papillae in their reciprocal tracts. Forty-six normal B feathers on S and fifty-two S feathers on B (a total yield of 74 per cent) were thus obtained by transplanting intact papillae, with some of the attached follicular wall and subdermal tissue, from one tract to the other (Lillie and Wang, 1941). In one operation, however, the bases of the transplants were trimmed under the dissecting microscope so as to rid them of all attached tissue. Eight such trimmed B and S papillae, each consisting solely of the dermal core and its epidermal covering, were then reciprocally transplanted. They yielded six normal B feathers on S and five S feathers on B. These differed in no way from those produced by untrimmed papillae in regard to structure, pigmentation, and capacity for subsequent regenerations. This indicates that all criteria of tract specificity are determined by the regeneration cells which constitute the epidermal component of a papilla. These cells evidently represent an inexhaustible source of successive generations of feathers.

When fully regenerated, these first-generation feathers from reciprocally transplanted B and S papillae, displaying their original donor tract specificities, were plucked. When the second generations had reached a length of some 40 mm., each duplicating its predecessor, they were again plucked. Papillae of second-generation feathers were used as a precaution in order that the first generation might insure the full consolidation of the tract specificity of the particular papilla. The active papillae remaining after the second plucking were then treated as follows: The follicle was slit open nearly to the bottom; a large drop of the denuding reagent (three to four times the volume used in the basic experiment) was spread over the papilla *in situ*, including the area around its base. After 1, 3, 5, or 7 minutes the resulting collodion film was removed without washing the affected area. The flaps of the follicular wall were closed as usual. As a rule, the treated papillae were slow in regenerating; the amount of delay was approximately proportional to the duration of the treatment. In a few cases as much as 6 weeks passed before the first sign of regeneration, while the most rapid cases took about 3 weeks.

The results of this series of experiments are given in Table 3. They seem to lack uniformity, apparently because of a new factor involved, the duration of exposure. After short treatment feathers of the same character emerged as in the preceding generations, while longer treatment increased the tendency toward reversal of the former tract character. Obviously, treatment of 1 minute failed to kill the epidermis of the papilla; hence the original donor tract specificity was preserved. Three minutes still permitted the recovery of the donor epidermis, al-

though in two cases successful partial killing of the epidermis with subsequent replacement from the host tract resulted in chimaera feathers which displayed both B and S features. Five minutes brought about complete destruction of the epidermal cells, together with serious damage to the dermal core, leading to defective feathers characteristic of the host tract as a result of the replacement of epidermis from the host tract. Seven minutes' exposure killed the entire papilla except in two cases, in which tuft feathers with host tract specificity were obtained.

The difference between the results of this experiment and those of the basic experiment seems to be primarily due to the different treatment. The papillae treated *in situ* had an advantage in that the vascularization was undisturbed. But an exposure of from 5 to 7 minutes killed not only the superficial cells but a substantial portion of the dermal core as well. This accounts for the structural defects of those feathers in which the treatment was sufficient to change the tract specificity. In the *in situ* experiment, as in the basic experiment, successful destruction of the epidermal component and subsequent replacement of local host epidermis were responsible for the observed alteration of tract specificity.

The two chimaera feathers in the last experiment (see Table 3) were obtained accidentally. Such cases can be produced deliberately by partial destruction of the epidermal component of a papilla either prior to its transplantation to the reciprocal tract or after it has been incorporated there. The latter was accomplished by the "hot-needle" experiment. Actively regenerating second-generation feathers, similar to those used in the *in situ* experiment, were plucked, and their follicles were opened as usual. While the papilla was held with forceps below its base, a heated steel needle was quickly applied to one side of the exposed papilla, chosen at random. Twelve B papillae incorporated in S and seventeen S papillae incorporated in B were so treated. The results of the experiments are given in Table 4.

The percentage of failures from the heat-treated papillae was high: eleven

TABLE 3

RESULTS OF THE *in situ* EXPERIMENT

Type of Original Feather	Expt. No. (Ser.)	No. of Papillae Treated	Duration of Exposure (Min.)	Resulting Feathers			
				Breast	Saddle	Chimaerae	Failed
S on B.....	46	7	1	0	4 normal, 3 defective	0	0
	48	5	3	0	4 defective	1	0
	41	4	5	1 tuft	1 defective	0	2
	43	4	7	1 tuft	0	0	3
B on S.....	30	5	1	4 normal, 1 defective	0	0	0
	31	6	3	2 normal, 3 defective	0	1	0
	37	6	5	0	4 tuft	0	2
	35	4	7	0	1 tuft	0	3

out of twenty-nine cases (38 per cent). Eight chimaerae (five on B and three on S) and ten nonchimaeric feathers were produced. Three of the chimaerae grown on B had the general shape of an S feather, but toward the base of the vane black-pigmented barbs predominated on one side. One interesting feature of all the chimaerae grown on B is the 180° spiral twisting of their axes. The twist may be ascribed to an immediate effect of the intense heat, since it entirely disappeared in the second generation. One of the three chimaerae grown on S was a better specimen in that the B and S components were more nearly equal. The B barbs appeared in one side of the vane, while the rest of the feather resembled an S feather (Pl. III, Fig. 12). The other four chimaerae (two on B and two on S) are principally B feathers with an S margin in the vane, evidently the result of random killing of the epidermis. Significantly, no chimaera of B margin on S vane was obtained (cf. Lillie and Wang, 1943).

The ten nonchimaeric feathers were all defective. The fact that some of them were B and others S regardless of the tract in which the treatment took place is significant. It indicates that the amount of epidermis killed was purely a matter of accident. Donor tract specificity was retained if the injury sustained by the epidermal coat did not involve the replacement by the epidermis of the host follicle, whereas manifestation of the host tract specificity was the result of a complete destruction of the epidermis followed by host replacement. In both cases the structural defects of the feather were presumably due to heat damage to the dermal core of the papilla.

The other method of production of chimaera feather was by partially immersing an isolated papilla in the denuding reagent, thereby achieving unilateral destruction of the epidermal component. The host follicle would be expected to replace only that fraction of the epidermis which had been destroyed, with the resulting production of a composite coat composed of both B and S cells. An isolated B or S papilla, after being rolled over a piece of filter paper, was placed, with its axis horizontal, in a drop of the denuding reagent on a small watch glass. The size of the drop was so adjusted that one lateral half of the papilla would protrude from the fluid and thus remain free from injury. The papilla was removed after 1 minute, washed in Ringer's solution, trimmed at the base, and transplanted in the usual manner to an empty follicle of the reciprocal tract. No effort was made to determine whether the immersed half was a dorsal, ventral,

TABLE 4

RESULTS OF THE "HOT-NEEDLE" EXPERIMENT

Type of Original Feather	Expt. No. (Ser.)	No. of Papillae Treated	Resulting Feathers			
			Breast	Saddle	Chimaerae	Failed
S on B.....	51	10	2 defective	1 tuft	3	4
	53	7	1 defective	1 tuft, 1 defective	2	2
B on S.....	54	6	1 defective	2 tuft	1	2
	57	6	1 defective	0	2	3

or lateral one. The results are given in Table 5.

The percentage of total failures was reduced to 24 per cent (seven out of twenty-nine cases), compared to 46 and 34 per cent, respectively, for the B → S and S → B grafts of totally immersed papillae in the basic experiments. This was probably due to the fact that none of the partially immersed papillae was killed by the treatment itself. The resulting feathers on either tract were of two distinct types: chimaerae and defective nonchimaeric feathers, the latter being invariably characteristic of the donor tract, in

TABLE 5

RESULTS OF THE PARTIAL-IMMERSION EXPERIMENT

Type of Transplantation	Expt. No. (Ser.)	No. of Papillae Treated	Resulting Feathers			
			Defective Breast	Defective Saddle	Chimaerae	Failed
S→B....	49	7	0	5	1	1
	50	7	0	4	0	3
B→S....	52	7	3	0	2	2
	55	7	4	0	2	1

contrast with the defective feathers characteristic of either the donor or host tract obtained in the "hot-needle" experiment. That there was no complete acquisition of host tract specificity is ascribable to the retention by the partially immersed papilla of half of its epidermal coat. The replacement of killed epidermis may take place either from host follicular epidermis or from cells of the intact papilla epidermis, these conditions giving rise to chimaerae and nonchimaeric feathers of donor tract specificity, respectively. The relative frequency of incidence of about 3 to 1 in favor of the latter indicates that there was a greater chance for the killed epidermis to be replaced by the surviving epidermal coat of the transplant than by the host follicle. This was later confirmed by an experiment in which dorsal B and S half-papillae were transplanted to empty follicles of reciprocal tracts. While all six such S transplants in B follicles gave rise to pure S feathers, two chimaerae were obtained on S from a total of twelve B grafts. There was, thus, only 13 per cent (two out of eighteen cases) in which the cut surface of the transplants became subsequently covered by the host follicular epidermis; in the remaining cases (87 per cent) the replacement took place from the cells of the graft.

All the chimaerae (see Table 5) that resulted from partially immersed grafts were free from twisting. This shows that the latter phenomenon is characteristic only of the heat treatment. The chimaera grown on B is slightly longer than a normal B feather; its vane, the apex of which is missing, is about two-thirds of the total length. On the left side of the vane the golden-brown pigment predominates except for a few black barbs near the base. On the right, B features predominate save for a distinct S margin. Such a chimaeric pattern is clearly accidental, unparalleled by any of the chimaerae produced by combinations of B and S half-papillae (Lillie and Wang, 1941, 1943). All the four chimaerae grown on S carry S margins in their vanes, two unilaterally and one bilaterally (Pl. III, Figs. 11, 13). They differ in total length, fluff proportion, and general form; but all are more or less intermediate between B and S.

All the chimaera feathers thus far produced, irrespective of the method used, bear additional evidence that tract specificity is determined by the epidermal component alone. They also demonstrate what the basic experiment failed to bring out, namely, that B and S regeneration cells, once they have been exposed to the influence of the dermal papilla, are capable of autonomous realization of their own tract specificity even when inter-

spersed with fragments of the other and regardless of the proportion of the two kinds of cells present in the mosaic epidermal component.

An experiment using picric acid as a denuding reagent was done before the salicylic acid mixture was established. Isolated papillae were totally immersed in saturated picric acid for 1 minute, thoroughly washed in Ringer's solution, and transplanted into follicles of the reciprocal tract. One capon served for the exchange of eight B and S papillae. The results from the eight S → B transplants were one good chimaera, which has been reproduced for five successive generations; five defective B feathers; and two cases of failure. The eight B → S grafts gave three chimaerae and two defective S feathers, while the rest failed. It is significant that the tract specificity of the defective feathers derived from both tracts were invariably those of the host tract. This indicated that the picric acid had completely killed the epidermal component in seven out of eleven cases, while the chimaerae were the result of replacement of partially destroyed epidermis of the graft. The latter resembled those derived from the partial-immersion treatment in that they were free from any twisting of the axis, but they were rather unique in the variation of structure in successive generations (see below). These results further substantiate the established fact that, whenever a composite epidermal coat is formed over a dermal core of a papilla, the resulting feather will be a chimaera displaying both B and S characteristics in proportion to the amount of the two epidermal cells which go into the making of the mosaic pattern.

3. *Successive generations of feathers.*—Special attention was given to the structure of the successive generations of the feathers resulting from each of the above experiments. Some of them are now in their sixth or seventh generation. The majority showed a persistent structural pattern with only insignificant variations in the type of defect—if any. In a limited number of cases, however, there was a general tendency toward (1) a progressive reduction in intensity of the defect and (2) a slight increase in the length of the feather (cf. chimaera feathers in Lillie and Wang, 1943). A few typical examples are given below: Experiment 107, No. 6, derived from a denuded S papilla transplanted to B, began as a typical half-vane B feather and remained so in the second generation, with only slightly increased barbs; but in its third generation it changed to a longer and almost perfect B feather, with just a few apical barbs missing (Pl. IV, Figs. 14, 15, 16). No. 5 of the same experiment was half-vaned in the first generation only; it became a relatively normal B feather in the second generation and has remained so. Four of the chimaerae from the picric acid experiment showed progressive changes in structure. Of the one grown on B, the first generation had severe apical defects; the right side of the vane was mostly S, while its left side carried only a spot of S margin. Its second and third generations were both about twice as long as the first generation, and with a much better developed S component in structure as well as in pigmentation (Pl. IV, Figs. 17, 18). The three chimaerae grown on S showed considerable increase in length in later generations, although no appreciable difference in structure. A similar tendency toward increase in the extent of the S component and total length of the feather was also manifested by the two chimaerae derived from partially immersed papillae (Expt. 55). These changes in structure, invariably in the direction of decreasing defectiveness, are believed to be due to some internal

readjustment within the injured dermal component of the papilla.

In addition to changes in structure and length, we have already noted (p. 335) the complete disappearance of axial twisting in second-generation chimaerae derived from the application of heat to papillae *in situ*. Another phenomenon commonly observed in tuft feathers was their increase in both length and number of barbs, although a shift from the tuft type to a less defective category (e.g., half-vaned) never occurred in later generations. This suggests that, although repair of damage is possible, apparently it has a limit.

These differences occurring in later generations have not hitherto been noted in feathers which resulted either from portions of intact papillae or from recombined papillae (Lillie and Wang, 1941, 1943) and hence must be regarded as significant. Apparently, there exists a difference between defective papillae involving loss of parts and those injured by means of heat or chemical effect. The results of the *in situ* experiment (p. 333) have given us some inkling as to the possible nature of this difference, although the real mechanism leading to the repair of the latter papillae is not clearly understood.

B. ROLE OF THE DERMAL COMPONENT

The technique of transplantation of denuded papillae was utilized to ascertain the seat of determination of symmetry in regenerating feathers. The results of the basic experiment were not adequate for this purpose, since all the denuded papillae used were oriented at random. Specially designed experiments were accordingly carried out on four capons. On each bird, twelve S papillae, treated with the denuding reagent by total immersion, were transplanted to empty B follicles with their axis rotated 180°, i.e., with the dorsoventral axis reversed. Six comparably treated B papillae, transplanted to their own follicles with similarly reversed axes, served as controls. Before the papillae were detached from their follicles, they were marked by a tiny notch cut at the dorsal apical end.[3]

Results of this series of experiments are presented in Table 6. The forty-eight S papillae yielded thirty B feathers on breast (including four tuft feathers which had no bearing on the question of orientation), while eighteen approximately normal B feathers were obtained from the twenty-four B papillae (62.5 and 75 per cent yield, respectively). The feathers were classified according to the position of the rhachis. Class 1 includes those feathers with the rhachis normally oriented in the median dorsal position or rotated by less than 45° in either direction. Class 2 represents those with the rhachis in the left quadrant; Class 3, those in the right quadrant. Class 4, with the rhachis in the ventral quadrant, comprises the feathers with completely reversed orientation.

The most striking result is that none of the feathers of which the denuded papillae had been rotated by 180° had a normal or near-normal orientation. Over 50 per cent showed the reversed orientation that corresponds to the inversion of the grafted papillae. The results are thus comparable to those obtained by Lillie and Wang (1941, p. 111), who used a totally different technique. They proved that the feather formed by the replacing host tract epidermis owes its orientation and symmetry to an orienting influence of the dermal core. What remains to be

[3] An attempt was made to cut the notch at the ventral side of the apex instead of the dorsal side, in order to avoid damaging the dorsal field; but it was unsuccessful because of the difficulties arising from the obscured view.

explained is the high incidence of moderately oriented feathers in Classes 2 and 3. It seems probable that these feathers arose as a consequence of deviations from the intended reversed orientation, which might have been due either to inaccurate marking or to a secondary shifting in the position of the grafts resulting from the bird's movements. At any event, these results confirm the conclusion of Lillie and Wang (1941) that the field of symmetry is located exclusively in the dermal papilla was transplanted into an empty follicle—the epidermis of the host follicle can supply an equivalent functional substitute. Thus, the results of the basic experiment alone provide sufficient indication that feather-forming capacity is at least not limited to the regeneration cells.

The problem then becomes: To what extent does the follicular epidermis as a whole possess feather-forming capacity? Is any part of it capable of feather regeneration in the presence of the indispensable dermal papilla, or is this capacity limited? The following experiments were designed to answer these questions.

TABLE 6

RESULTS OF TRANSPLANTATION OF DENUDED PAPILLAE, 180° ROTATED

Orientation of the Resulting Feathers	Structure						
	From Denuded S Papillae					From Denuded B Papillae	
	Approx. Normal	Missing Apex	Half-Vane	Apical Tuft	Per Cent	All Approx. Normal	Per Cent
Class 1 (dorsal quadrant)	0	0	0	0	0	0	0
Class 2 (left quadrant)	2	3	2	0	27	5	28
Class 3 (right quadrant)	4	0	1	0	19	3	16
Class 4 (ventral quadrant)	5	7	0	1	50	10	56
Undetermined	0	0	0	1	4	0	0
Total	11	10	3	2	100	18	100

mal component of the papilla. With this finding, the morphogenetic roles of the two components of the papilla are thus clearly defined.

C. FEATHER-FORMING CAPACITY OF FOLLICULAR EPIDERMIS

Lillie and Wang (1941, pp. 109, 130) regarded the regeneration cells (p. 325, n. 2) as totipotent so far as the feather is concerned. This view is further substantiated by the experiment described on page 333. It was shown above (p. 332) that the regeneration cells, when removed, could not be replaced by the remaining follicular epidermis in the absence of a dermal papilla. But, when the latter is present—e.g., when a denuded

In the first experiment denuded active S papillae were transplanted to different levels of the follicular wall of empty B follicles. A small hole, about the diameter of a papilla, was made on the ventral or dorsal wall of the recipient follicle without removing any of the incised tissue. The graft was placed well into the hole with its base securely anchored (see text Fig. 1). A total of sixteen denuded S papillae were transplanted each into a separate follicle with the center of the graft located at approximately the 1-, 2-, 3-, or 4-mm. level, measured from the

bottom of the follicle. From the sixteen grafts (four at each of the four levels) two feathers were obtained at the 1-mm. level, one each at the 2-mm. and 3-mm. levels, but none from the 4-mm. level.

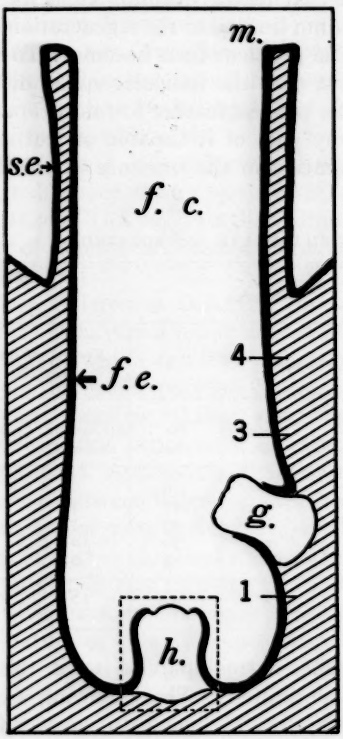

Fig. 1.—Diagram of a longitudinal section of a breast follicle showing a denuded saddle papilla (*g.*) grafted to its wall 2 mm. from the bottom. The host papilla (*h.*) may be left intact or removed; the latter condition is indicated by the dotted lines. Subdermis is represented by oblique parallel lines. *Key: f.c.* = follicular cavity; *f.e.* = follicular epidermis; *s.e.* = skin epidermis; *m.* = mouth of the follicle; 1, 3, and 4 indicate the 1-mm., 3-mm., and 4-mm. levels at which similar grafts were implanted.

All four feathers were typical B feathers, similar to those obtained from the basic experiment. As controls, four untreated S papillae were similarly transplanted to each of these four levels; and, in addition, another four to the remaining apical portion of the follicular wall. Comparable results were obtained but with better yield: three S feathers at the 1-mm. level and two at each of the next three levels, while none was produced at the most apical portion. Since normal feathers were obtained from denuded papilla-grafts implanted anywhere up to the 3-mm. level, it would seem that the follicular epidermis as high up as that level has a feather-forming capacity equal to that of the regular regeneration cells.

Production of feathers at these same levels of the follicle was next attempted by the implantation of denuded papillae into follicles, the papillae of which were retained intact (text Fig. 1). This experiment was believed to be crucial, since, if successful, two wholly independent feathers would grow in the same follicle. Altogether, nineteen operations were made on a total of four birds, with either the dorsal or ventral follicular wall used as a site of implantation. The results of this series of experiments are listed in Table 7.

In each of the three successful cases two entirely independent feathers were obtained. The one derived from the graft showed certain defects, and its orientation bore no relation to that of the feather derived from the intact host papilla. For instance, in the one obtained on the White Leghorn hen (Table 7) the two feathers faced each other with their dorsal surfaces, a condition which never occurs in twinning resulting from operation (Lillie and Wang, 1941). In the two cases on the Brown Leghorn capon, on the other hand, the feathers were oriented with the ventral side of one facing the dorsal surface of the other (Pl. IV, Fig. 19). Since the two partners are attached at their calami, the distance between the bases of the calami (inferior umbilici) should express the position of the graft relative to the normal papilla. This was actually so for the host and donor papillae in the case on the White Leghorn hen.

They were found, upon dissection, to be exactly 2 mm. apart from each other. These results prove that the follicular epidermis in question can, under the stimulus of the dermal graft, form a feather not only in the absence of the host papilla but even while the latter is engaged in normal regeneration.

Finally, experiments were devised to test whether or not extrafollicular epidermis has feather-regenerating capacity when provided with a dermal papilla. The region directly above the breast is ideal for this purpose because of its rich vascularization and the protection offered by the wing. A row of small cuts about 2.5 mm. long and 4 mm. apart were made on the skin along the course of a sizable blood vessel. Just prior to transplantation the skin openings were slightly enlarged along their edges, and all the subdermal tissue directly underneath was removed. The opening was then moistened with Ringer's solution, and a denuded papilla was transplanted into it with its base firmly placed against some exposed capillaries; the whole graft was finally covered up with the incised skin fold. For 2 hours the grafts were continually moistened with Ringer's solution, while the bird remained under anesthesia. After that, in order to prevent the grafts from drying, a watch glass was inverted over the operated area with its edge securely fastened to the skin by a Scotch tape. The bird was caged alone, and an effort was made to see that the watch glass remained in place for at least 4 days.

Two Brown Leghorn capons were used in one experiment. One of them carried twelve denuded active B papillae, and the other, nine denuded active S papillae, transplanted into skin openings above the breast. The result was entirely negative: none of the transplants yielded any feather. Within 1 week the skin wound closed and the grafts appeared to have dried up.

TABLE 7

RESULTS OF GRAFTING DENUDED PAPILLAE TO THE FOLLICULAR WALL OF NORMAL FOLLICLES*

Level of Grafts	Bird Used	No. of Cases	Donor Tract	Host Tract	Position of Grafts	Successful Cases
1 mm.....	Brown Leghorn capon	2	S	B	dorsal	1
		2	B	B	ventral	1
2 mm.....	White Leghorn hen	5	B	B	dorsal	1
3 mm.....	Brown Leghorn cock	5	B	B	dorsal	0
4 mm.....	Brown Leghorn cock	5	B	B	ventral	0

* A case was considered successful when two feathers developed from one follicle.

For a control another capon was similarly operated on, with the transplantation of six intact active B papillae and two active S papillae, both types of papillae being untreated. Five days after the operation both of the S grafts and four of the six B papillae were found to have commenced regeneration. These grafts appeared to have resumed regeneration very quickly, apparently as a result of rapid re-establishment of vascularization. At the end of a month three of the four B feathers had formed normal follicles, while the fourth one was not growing equally well. Of the two S feathers, one was healthy, with a completely developed follicle, whereas the other had shown signs of regression. The net outcome was, therefore, one S and

three B feathers, which are now in their sixth generation.

The contrast shown by these two experiments in regard to the capacities of denuded and intact papillae to produce feathers may be indicative of an inability of the extrafollicular skin epidermis to respond to the dermal component of the papilla, although mechanical or other local conditions arising from the absence of the protective epidermal lining, as well as of the follicle, may have caused the denuded grafts to disintegrate.

The potentiality of the apical half of the follicular epidermis has been left undetermined, owing to difficulties involved in the operation. Whatever it may be, the results of these experiments seem to favor the conclusion that the feather-forming capacity is retained by the definitive follicular epidermis only.

IV. DISCUSSION

Feather regeneration as an induction system.—The feather is a product of active collaboration between the epidermal and dermal constituents of the papilla. The two components are mutually indispensable, since neither can produce a feather alone. Specifically, then, what does each of them do? Obviously, the epidermis furnishes the building material, while the dermal papilla sets this material in operation as well as directs its organization. The action of the dermal papilla has been described as having the attributes of a "field" (Lillie and Wang, 1941). The fact that no feather can be formed in the absence of a dermal papilla shows that the latter imparts a specific activation to the epidermal cells, without which these cells would only remain "idle." The field next induces a "secondary" field in the developing epidermis in correspondence with its own polarity by initiating the formation of a series of parallel barbs dorsally, whereby the position of the future rhachis is determined (Lillie and Wang, 1941, p. 106). Concurrently with the establishment of the symmetry of the feather, or prior to it, the epidermis becomes a self-differentiating system. Defective papillae lacking a complete field (Lillie and Wang, 1941) or those injured by heat and chemicals lead invariably to structural defects either as a result of deficient field action or indirectly due to upset of the developmental mechanism, such as, for instance, the derangement of the tangential movement of barbs. It is thus clear that, although the feather is differentiated from the follicular epidermis, its symmetry and orientation are, nevertheless, under the control of the dermal field. Aside from these morphogenetic properties, the dermal papilla also performs important physical and physiological functions (Lillie, 1940) in providing a scaffolding for the support of the feather cylinder and the continual production of pulp throughout the period of regeneration as a means of distributing nutrition to the growing cells of the feather.

The dermal papilla, however, apparently does not impart any new characters to the epidermal cells but merely transforms them into whatever the latter are already intrinsically prepared to be. We have seen, for instance, that the denuded breast and saddle papillae, i.e., pure dermal components, do not differ in their organizing properties, since they can be interchanged to collaborate with the epidermis of the other tract without affecting the tract specificity of the regenerate derived from such a composite papilla. This means that, whereas the influence of the dermal component is of a general nature and acts presumably on both kinds of follicular epidermis in the same manner, the response from the latter is, in contrast, very specific. For the breast

epidermis produces only breast feathers, regardless of whether it is provided with the dermal papilla of its own or of another tract; and, similarly, saddle epidermis produces only saddle feathers. In general, then, feather regeneration is in line with many known developmental systems in early embryogenesis (Weiss, 1939, pp. 321–437), in which a mesodermal tissue incorporates and effects the differentiation of an overlying ectoderm. While, in such a dual system, the ultimate character of the organ in question is determined by the responding ectoderm in accordance with its potency, the polarity, symmetry, and the normal developmental mechanism of the differentiating organ come under the influence and control of the mesoderm. Tissues endowed with the latter properties, to which the dermal papilla evidently belongs, are often referred to as "inductors." Experiments now under way in this laboratory which are directed more specifically to localizing and delineating the papilla-inductor (see Lillie and Wang, 1944) may soon yield more definite information regarding its nature.[4]

The question arises as to wherein lie the ultimate differences between the epidermis of the breast and the saddle, respectively. The cells themselves do not appear to differ morphologically. Yet they must differ physiologically, since, faced with the same dermal stimulus, one type develops into breast, and the other into saddle, feathers, which, as we know, differ specifically in their growth rate, shape, pigmentation, and the structure of barbs. These differences, which constitute the so-called "tract specificities," must be ascribed to the specific properties that each epidermal region acquired during its embryonic differentiation. One recalls the work of Weiss and Amprino (1940), who demonstrate that perfectly homogeneous and morphologically indistinguishable prescleral mesenchyme of 4-day chick embryos, when cultured *in vitro*, produces cartilage of the specific scleral variety. The potencies of any tissue or cells, therefore, cannot and should not be judged by their appearance but may be learned only from the outcome of their performance.

Possible relationship between the differential growth rate and pigment production of feather tracts.—We do not know whether the persisting differences in the growth rate of breast and saddle feathers can be traced back to correspondingly different growth potentials of their respective follicular epidermis or whether the difference represents something acquired during the course of differentiation. Much significance has been attached to it in the interpretation of hormone threshold (Lillie and Juhn, 1932; Juhn, Faulkner, and Gustavson, 1931; Juhn and Gustavson, 1930) and in the determination of the rhythmic pattern in the Barred Plymouth Rock feathers (Montalenti, 1934; Willier, 1941). A possible correlation between basal metabolism and growth rate of feathers is demonstrated by Lillie and Wang (1940). Recently, the latter authors (1943) showed that in a certain composite collar from combination of breast and saddle half-papillae the observed replacement of saddle cells by those of the breast might be due to some intrinsic property, possibly again related to their higher growth rate.

Pigment cells have been demonstrated to be of neural-crest origin in both amphibians and birds[5] (Du Shane, 1938, 1939; Dorris, 1939; Eastlick, 1939*a* and 1940; Danforth, 1937, 1939; Hamilton, 1940, 1941; Rawles, 1939 and 1940*a*;

[4] See Lillie (1942) for a review of the recent work on feather development and for the complete citation of its literature.

[5] Rawles (1940*b*) demonstrates the neural-crest origin of melanophores in the mouse also.

Ris, 1941; Watterson, 1941, 1942; Willier and Rawles, 1940). Attention is now centered on the specificity of melanophores and the physiology of production of pigment. The unsettled issue in the dimorphic Brown Leghorn fowl is whether in the male (or castrates of both sexes) there are two types of melanophores for the breast and saddle, respectively, or whether only one type is involved in the production of both the black and the brown pigments, possibly under different conditions of the substratum (epidermis).

The indication from the present results seems to favor the latter theory. We have seen that feathers derived from untreated papillae retain the pigmentation of their original tract irrespective of the site of growth, whereas those from denuded papillae exchanged between breast and saddle invariably take on the pigmentation of the host tract. What does this contrast imply? It suggests that, whatever their source, the melanophores appear to be dependent in some specific manner upon the feather-forming epidermis. The feathers derived from the untreated grafts retain the pigmentation of their tract of origin because the entire papillae survive, while those derived from the denuded papillae become pigmented in accordance with the host tract because their new epidermal component is of the latter origin. Clearly, the epidermis is the controlling factor: without exception, the pigmentation follows the tract which contributes the epidermal component of the papilla, whether in normal regeneration or following grafting. In short, the pigment is determined concurrently with the other morphological criteria of tract specificity.

The situation is analogous to the "dependent" melanophores of the white axolotl (Du Shane, 1935, 1939). In this unpigmented axolotl the melanophores are present but will not produce melanin in the absence of a specific substance; the latter, though normally lacking in the animal itself, can be supplied by epidermis taken from a pigmented species such as *Amblystoma punctatum*. Earlier, Harrison (1935) found a similar situation in the limb of *Triturus torosus* embryos. As to the nature of this substance present in the epidermis necessary for the production of specific pigment by the potential melanophores, Du Shane (1935) suggests a specific oxidase (dopa oxidase) comparable to that found in the mammalian epidermis originally investigated by Bloch (1917).[6]

In case of the follicular epidermis of the fowl under consideration, a certain physiological provision necessary for the oxidation of dopa into black melanin appears to be lacking in the saddle follicles. This might be due to the production of a substance by the saddle epidermis, which acts as an "inhibitor" to the synthesis of black melanin (cf. Dorris, 1939), or it might be that the chain reaction effecting the oxidation of dopa into melanin comes to a halt at an intermediate stage at which the oxidation product is a brown pigment, such as is characteristic of the saddle feather (cf. Dorris, 1942, p. 117). The latter alternative may be the result of a mere quantitative difference—for instance, in the oxygen content of the oxidases. In any event, we assume that the undifferentiated melanoblasts of the two tracts are physiologically the same and hence produce the same propigment, dopa; the determination as to which of the two (black or brown) melanins the latter will form upon oxidation lies with the intrinsically different properties of the follicular epidermis of the two tracts. The fact that the breast and saddle differ in the growth rate of their feathers leads one to surmise

[6] For recent discussion on this subject see Du Shane's review (1943).

that the ultimate cause for the differentiation of pigments might have arisen from a difference in the metabolic rate of the cells. This hypothesis presupposes, of course, the existence of a certain threshold, and the rates above and below it are responsible, respectively, for the black and brown pigments. If the growth rates of breast and saddle feathers (Table 1) represent the metabolic rates of their respective follicular epidermis, a direct correlation may be drawn to attribute the different pigments to their respective growth rates. But, as yet, no such demonstration is available.

A point of important bearing on pigmentation concerns the source of melanophores in the feather germ. Willier and Rawles (1941, p. 195) originally suggested that probably the regeneration cells represent the source of reserve melanophores. Although our results do not indicate which of the two components of the papilla may be their permanent residence, they do render it doubtful that the mesoderm could be a source of chromatophores in feather regeneration. The question will be decided by the experiments of Foulks, whose preliminary report (Foulks, 1942) revealed that "melanoblasts arise outside of the feather germ and penetrate the collar after migrating through the dermal papilla." This seems to imply that neither the dermal nor the epidermal component permanently harbors the pigment cells but rather that the melanoblasts migrate anew in each regeneration from the subdermis into the collar by way of the dermal core. Significantly enough, this finding helps one to explain how trimmed papillae reciprocally transplanted between breast and saddle tracts could furnish the donor-tract pigment without exhaustion (p. 333). It is probable that a new supply of local (host) melanoblasts are utilized in later generations which are of the same physiological type as those of the donor tract. Such an interpretation favors the "monophyletic" theory.

The above considerations conform with the works of Hamilton (1940, 1941), who stressed the autonomy of black and red melanophores in the red breeds of fowl studied *in vitro*. Our results agree with his statement that "a precursor cell might be incorporated in the feather germ and then diverge either in the black or red direction *in situ* due to physiological differences or external conditions in its environment" (Hamilton, 1941, p. 295; Dorris, 1942, p. 117); but, in addition, our results point definitely to the follicular epidermis as constituting perhaps the primary factor implicated in the differentiation of the two types of melanophores (cf. Willier, 1943, pp. 141, 142). A slight divergence of opinion concerns the mode of action of hormones: While the cultivation of relatively free melanoblasts *in vitro* perfectly justified Hamilton (1941, p. 296) in concluding "that hormones are not acting secondarily through some intermediate cell type" when definitive follicular tissue is not present, this statement may not necessarily apply to hormones when the latter are allowed to come into direct contact with the follicular cells *in situ*. This concept receives indorsement from Willier (1943, p. 141) in his interpretation of Hamilton's work. The induction of female growth rate in the capon (Juhn, Faulkner, and Gustavson, 1931, p. 78) simultaneously with plumage feminization caused by the injection of female sex hormone speaks favorably for the possibility that hormones may exert their specific effect on melanin synthesis through the medium of follicular epidermis.

Reinterpretation of related work having a bearing on the epidermal origin of tract

specificity.—Eastlick (1939*b*), in his study of feathers obtained from limb transplants made between embryos of different breeds of fowl, concluded that invading host mesoderm was probably responsible for the mixed host and donor types of the feathers as well as for their growth rate. In the light of the evidence presented above, this could hardly be the case; instead, it must have been due to the actual incorporation of host follicular epidermis in the transplants. This interpretation is in full accord with the results of Willier and Rawles (1940). In all their cases the transplanted skin and pure mesoderm alike supplied the specific donor melanoblasts to the graft region, while the follicles, including entire papillae were of host origin. Consequently, the resulting feathers invariably have and retain the form, structure, and growth rate of the host, while their pigment is produced by donor pigment cells until the time of their replacement by host melanophores..

The prolonged maintenance of donor color pattern in the mosaic feathers resulting from skin grafts (Danforth and Foster, 1929; Danforth, 1937, 1939) has not been fully accounted for, although Willier and Rawles (1940, p. 125) already correctly pointed to the different techniques used by them and by Danforth as the fundamental cause. More specifically, in Danforth's experiments the skin grafts carried over not only abundant melanoblasts but with them also complete follicles. During the operation certain papillae along the path of the scissors were probably incised; and, by chance, these split papillae of host and donor origin might have subsequently fused and healed together, thereby giving rise to composite papillae.[7] Another alternative was the formation of composite papillae by incised papillae of the graft and host follicular epidermis, or vice versa, along the line of healing. This is more probable than the actual fusion of part-papillae of both the host and the graft, in view of the improbability of bringing together, by chance, in one follicle cut papillae of both origins. One recalls (p. 336) that chimaera feathers of such a derivation were actually experimentally produced. In any event, only by either of these two modes of origin could we explain the fact that the mosaic feathers occurred only at the margins of the graft, never in the graft itself or in the purely host area. There is no doubt that such mosaic feathers incorporated the genetically specific host and donor types of melanoblasts as has been proved histologically to be the case (Danforth, 1937, p. 463). But, if the migration of the pigmentoblasts alone was responsible for the mosaic character of the color pattern in these feathers, a replacement of the donor melanophores by those of the host was not only certain to occur but should take place sooner than in the feathers obtained by Willier and Rawles (1940). The persistence of the mosaic pattern in Danforth's feathers must, therefore, be explained on a material basis, namely, that the host and donor melanophores are each accompanied by its own epidermal complement of the same origin. The mosaic feathers are thus comparable to the chimaerae produced by combination of parts of papillae (Lillie and Wang, 1941, 1943), in that the composition of their papillae remains stable, once it is established. Unfortunately, however, no reciprocal grafting of skin was made between tracts of contrasting morphology, such as, for instance, between the breast of the Barred Plymouth Rock and the saddle of the male Leghorn. The latter ex-

[7] This was the original explanation of Danforth and Foster (1929, p. 465), but it was later abandoned by Danforth (1939, p. 173).

periment would be a crucial test for the ventured interpretation of the origin of the mosaic feathers.

Differences in the nature of defective papillae and their consequences.—Finally, the observed structural changes in later generations of the experimental feathers, which, so far, constitute the first exceptions to the usual maintenance of structural patterns in feathers derived from defective papillae (Lillie and Wang, 1941) and those from combined half-papillae (Lillie and Wang, 1943), call for an explanation. To understand this contrast, one may turn to the *in situ* experiment (p. 333). There we have noted the importance of the duration of exposure: a duration which would have been sufficient to kill the epidermis of isolated papillae proved to be without effect when applied to papillae treated *in situ*. This indicated that the chemical effect from 1 minute's exposure was perhaps not instantaneously lethal to the tissue, particularly the dermal core, but that its destruction would ensue when subsequently followed by a period of lack of vascularization, as happened to many of the denuded grafts in the basic experiment. The papillae treated *in situ*, however, retained their normal vascularization and thus required several times the normal duration of exposure in order to be killed. A shorter duration resulted in complete or partial recovery of the affected papilla. Thus, it appears possible that cells subjected to heat or chemicals may remain quiescent and functionless, i.e., dormant, for a certain length of time; and, since the dermal core sustains relatively less injury than the superficial epidermal coat in any treatment, the chance for recovery in the dermal core is greater. This is quite in line with our observation that most of the changes in later generations concerned length of the feather and kind of structural defects—which, as we know, are expressions of the relative intactness of the field in the dermal papilla—and, further, that the changes are always in the direction of diminishing defectiveness. The increase in length or improvement of structure never extend beyond the third generation, a fact which lends support to the idea of a gradual but limited recovery from damage sustained during the treatment.

Needless to say, the ability to repair temporary injury is limited to only those papillae which do not suffer material losses from actual removal. Portions of a papilla remaining after an operation, owing to lack of power to replace the missing part, will remain as defective as they were at the beginning; and hence the structural pattern of the feather it produces remains invariable too.

Other possible causes for the structural variations are mechanical in nature. A physical hindrance to the developmental process of the feather, such as might be caused by the presence of a scar on the surface of a healed graft or by prolonged adherence of debris to the incorporated graft, may so derange the regular mechanism of feather development as to produce defects in the first generation which otherwise would not be formed. Such being the case, the better-developed later generations would naturally be the result of subsequent removal of the physical obstruction.

V. SUMMARY

1. Morphologically contrasting breast and saddle tracts of Brown Leghorn capons were used to separate the relative roles of the epidermal and dermal components of the papilla in the determination of tract specificity of regenerating feathers.

2. Isolated papillae were immersed in a solution consisting of 25 gm. of salicylic acid and 5 gm. of camphor dissolved in

100 cc. of Collodion Flexible (Merck) for 1 minute, to destroy their epidermal coat.

3. When breast or saddle papillae, completely denuded of their epidermal component, were autoplastically transplanted to empty follicles of the reciprocal tract, they invariably produced feathers characteristic of the host tract in growth rate, shape, form, structure, and pigmentation.

4. Histological study of the grafts revealed that the process of replacement of dead graft epidermis by epidermis of the host follicle was completed on about the sixth day following implantation. The possibility of substitution of the whole grafted papilla by host material was ruled out by (*a*) histological evidence and (*b*) implantation of small pieces of pulp and isolated follicular subdermal tissue into empty follicles, neither of which provoked formation of a new papilla. The composite character of the experimental papilla, consisting of the dermal core of the donor tract and a newly acquired epidermal coat from the host tract, has thus been definitely established. The change in tract specificity of the denuded papillae must be ascribed to their having become recoated from the reciprocal tract.

5. Essentially the same results were obtained by denuding papillae *in situ* after they had been well established following reciprocal transplantation. Full host tract specificity was acquired only in cases in which the original epidermal component had been completely destroyed. Similarly transplanted papillae, when partially denuded by heat injury or partially immersed in the denuding reagent, produced chimaerae with mixed breast and saddle characters, in addition to various types of defects.

6. It is concluded, therefore, that tract specificity is a function of the epidermal component of the papilla alone. The dermal components of breast and saddle are not tract specific.

7. It is thus revealed that epidermal cells of different regions, in spite of the absence of morphological and histological distinctions, differ in their morphogenetic properties and, hence, give rise to different products in response to the same stimulus.

8. Denuded papillae, transplanted in random dorsoventral orientation, give rise to feathers of corresponding orientation. When rotated 180° at the time of implantation, most of the resulting feathers were inverted. This demonstrates that the factors determining the symmetry of the feather reside in the dermal component.

9. The capacity to form a feather was found to be present at least in the entire basal half of the follicular epidermis. Extra-follicular (skin) epidermis, however, could not be demonstrated to possess this capacity.

10. The role of the dermal component and the bearing of the present results on a possible relationship between the follicular epidermis and pigment production are discussed.

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PLATE I

FIG. 1.—Normal breast feather of Brown Leghorn capon. Dorsal view. $\times\frac{3}{4}$.

FIGS. 2–5.—B feathers from denuded S papillae transplanted to B follicles, representative of the results of the basic experiment. From left to right: approximately normal (Expt. 34, No. 4), missing apex (Expt. 47, No. 2), half-vane (Expt. 129, No. 1), and tuft, or rhachisless (Expt. 125, No. 2). Dorsal view. $\times\frac{3}{4}$.

PLATE II

FIG. 6.—Normal saddle feather of Brown Leghorn capon. Dorsal view. $\times\frac{3}{4}$.

FIGS 7–10.—S feathers from denuded B papillae transplanted to S follicles, representative of the results of the basic experiment. From left to right: approximately normal (Expt. 38, No. 5), defective apex (Expt. 34, No. 1), half-vane (Expt. 127, No. 4), and apical tuft (Expt. 38, No. 2). Dorsal view. $\times\frac{3}{4}$.

PLATE III

FIG. 11.—Chimaera feather from a partially denuded B papilla transplanted to S follicle (Expt. 55, no. 3). It resembles an S feather in length and fluff proportion and carries a "lacy" (S) margin on both sides of the vane; but the entire apex (black) and the greater central portion of the vane are B. Dorsal view. Natural size.

FIG. 12.—Chimaera feather from a heat-treated B papilla after its establishment in an S follicle (Expt. 57, No. 4). It retains the length and fluff proportion of a B feather, while its complete right vane-half and a narrow margin on the left are S. Dorsal view. Natural size.

FIG. 13.—Chimaera feather from a partially denuded B papilla transplanted to S follicle (Expt. 52, No. 3). Except for a large S portion in its left vane-half and its increased total length, the rest of the feather is B. Dorsal view. Natural size.

PLATE IV

FIGS. 14, 15, AND 16.—First-, second-, and third-generation feathers, respectively, from a completely denuded S papilla transplanted to B follicle (Expt. 107, No. 6). These feathers, completely B, show continual decrease in the intensity of structural defects (see p. 337). Dorsal view. $\times\frac{4}{5}$.

FIGS. 17 AND 18.—First- and second-generation chimaerae from an S papilla treated with picric acid and transplanted to B follicle (see p. 337). The S characters are represented by only a few S barbs in the right vane-half in the first generation, but they make up the entire right vane-half, as well as a margin on the left, in the second generation. Dorsal view. $\times\frac{4}{5}$.

FIG. 19.—Two feathers (see p. 340) grown in the same B follicle. The one at the left is from the host papilla; that at the right from a denuded S papilla transplanted to the dorsal wall of the follicle 2 mm. up from its bottom. Their orientation, one on the top of the other, is accidental. Dorsal view. $\times\frac{4}{5}$.

PLATE V

FIG. 20.—Longitudinal section of a denuded S papilla in a B follicle, 2 days after implantation. Approximately the basal third of the follicle is shown (Figs. 20–22). Epidermis of the host follicle has covered the graft, on its right, to the level indicated by the arrow, while on its left no replacement has begun, although the supplanting epidermal sheet has already made the upward turn (*arrow*). On this side the graft is bounded by a darkly stained streak which consists mostly of remnants of dead graft epidermis. Necrosis has not set in; the apex of the graft is fused with the blood clot in the follicular cavity formed at the time of implantation. Heidenhain's Iron-Hematoxylin stain; 8 μ. $\times$95.

FIG. 21.—Longitudinal section of a denuded S papilla in a B follicle, $4\frac{1}{2}$ days after implantation. Replacement of host epidermis has advanced much further (*arrows*). The graft is necrotic particularly at the apex, which is crowded with islands of dead epidermis intermingled with host lymphocytes and other myeloid tissues (note a large cluster at the right). Delafield Hematoxylin and eosin stains: 8 μ. $\times$95.

FIG. 22.—Longitudinal section of a denuded S papilla, 7 days after implantation to a B follicle. The graft, having survived the necrosis, is now completely covered with epidermis. Nevertheless, its base remains clearly demarcated from the host subdermis. The entire graft (dermal) appears much healthier than in the earlier stages. The small vacuole-like structures at its very apical end immediately beneath the newly formed epidermal lining represent spaces formerly occupied by the remnants of graft epidermis; one such island is shown (*arrow*). The darkly stained structures filling the follicular cavity right above the incorporated graft consist chiefly of debris, including remnants of the blood clot and keratin fibers formed by the outer layers of the follicular epidermis. Heidenhain's Iron-Hematoxylin stain; 8 μ. $\times$95.

PLATE I

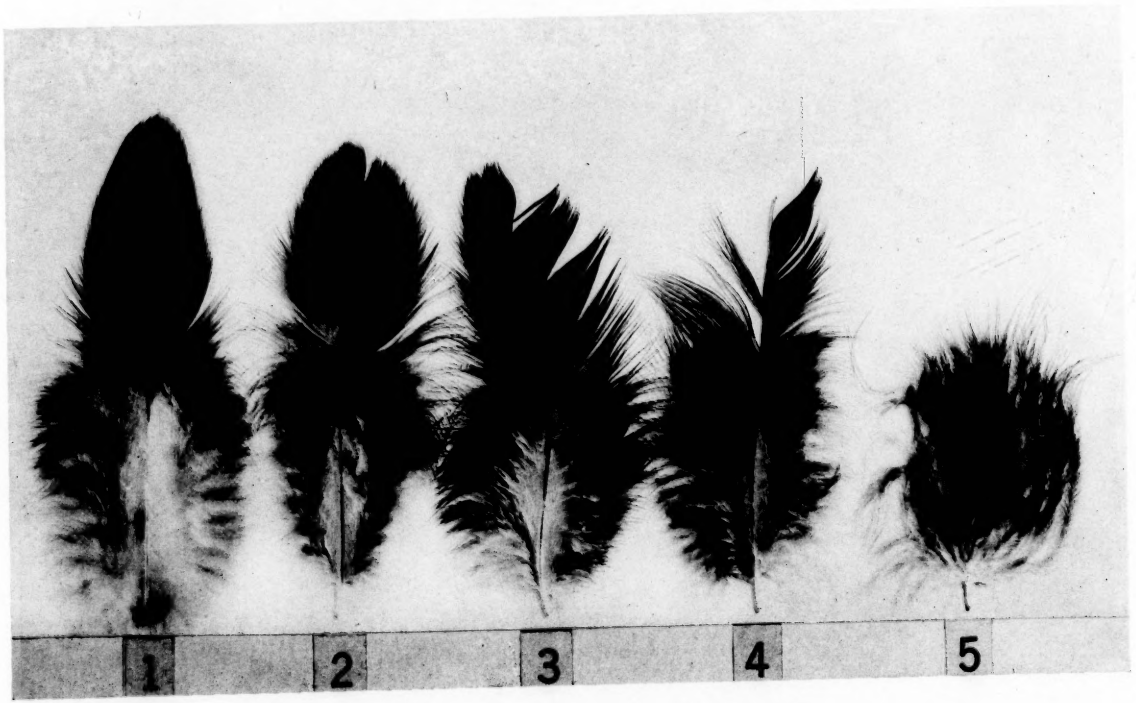

PLATE II

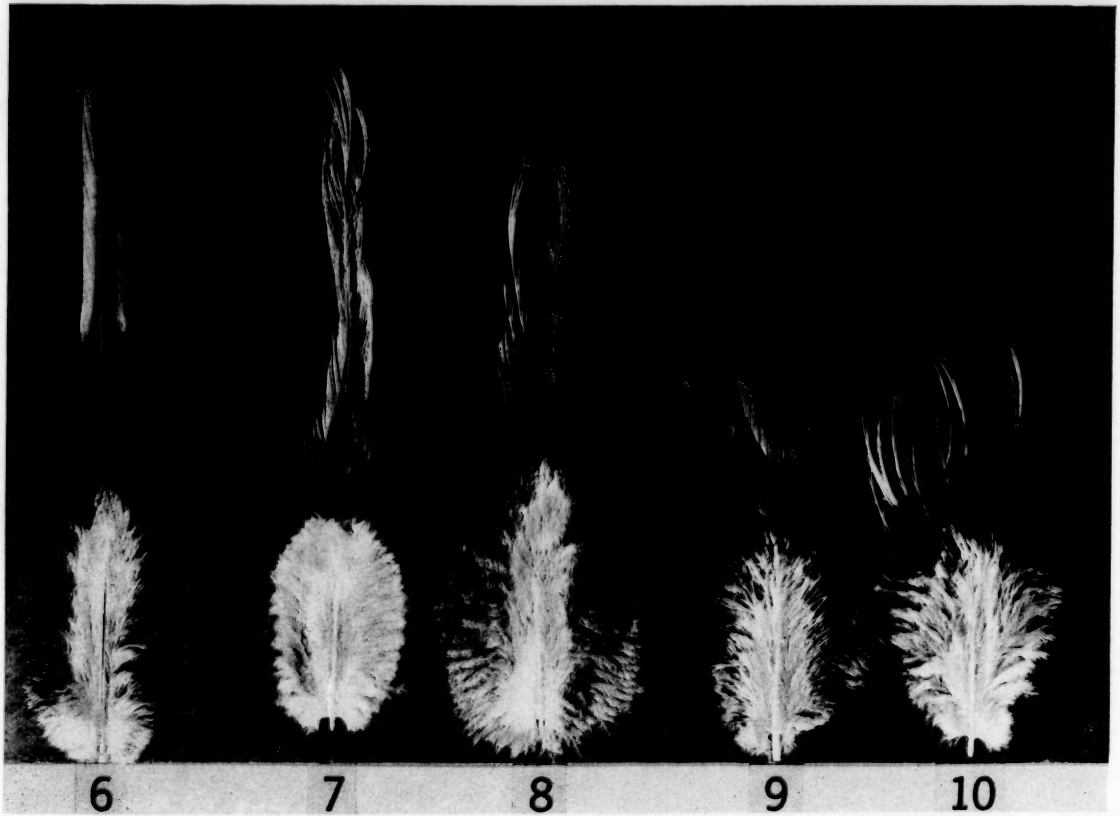

PLATE III

PLATE IV

PLATE V

AN ANALYSIS OF THE SOURCE OF MELANOPHORES IN REGENERATING FEATHERS[1]

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I. INTRODUCTION

THE color patterns which often characterize the skin of vertebrates have been the object of a number of investigations designed to analyze the developmental mechanisms responsible for their formation. Coloration may be produced in a number of ways, but in the majority of cases melanin pigment is the primary color constituent. Granules of melanin pigment are synthesized by special chromatophores, which typically reside in the epidermis and its structural derivatives but may also occur in the derma of the skin and in the coelomic epithelium. The source of melanophores and the factors governing their function and distribution have long been problems of considerable biological interest. A wide variety of histological observations, involving embryonic and adult tissues of every class of vertebrates, have been brought to bear upon the general problem of melanophore origin. The majority of earlier workers concurred in the general conclusion, formulated by Aeby (1885), that "no pigment is formed in the epithelium, but it is brought in by wandering cells from the neighboring connective tissue." However, in certain material (particularly feathers) this relation was not apparent, and some authors maintained that the melanophores appearing within the epidermis arise *in situ*, either from indifferent epidermal cells or from special melanoblasts. A few observers have also postulated that ordinary epidermal cells are capable of synthesizing pigment entirely independent of any special pigment cells—so-called "free" pigment formation. One author (Lloyd-Jones, 1915) has even contended that the entire coloration of the feather can be accomplished in this manner. The literature on this subject is extensively reviewed by Post (1894), Strong (1902), Biedermann (1928), and Watterson (1942).

The early investigators were principally concerned with determining the germ layer from which the melanophores take their origin. The controversy which developed is primarily of historical interest. Recently, precise experimental studies have clearly demonstrated the mode of origin of the melanophores in the embryos of Amphibia (Dushane, 1935 and 1938) and birds (Dorris, 1939; Eastlick, 1939; and Ris, 1941) and suggest that the same process occurs in mammals (Rawles, 1940). Prospective melanophores (melanoblasts) arise from the neural crest and are distributed to all parts of the body surface by extensive migration during early stages. In order to participate in feather pigmentation, these cells must eventually make their way into the epidermal primordia of the feather elements. The developmental

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history of this process in the chick embryo has been clarified by the careful histological description of Watterson (1942). From this work it appears that the pigmentation of the down feather is due solely to descendants of melanoblasts which penetrate the epidermis during a limited interval in embryonic development (eightieth to ninety-first hours in the case of wing skin). However, Rawles (1942) has shown that the migratory activity of melanoblasts continues and their penetration of the epidermis may be resumed, at least during the post-hatching period. Juvenile feathers, developing from skin which has been deprived of its normal source of pigment cells and transplanted after hatching to the back of a pigmented host, may become pigmented owing to a migration of host melanophores into the grafted skin. However, if the host is from a white breed, the feathers of the graft remain permanently unpigmented, conclusively disproving the theory of "free" pigment formation with respect to feather coloration. When pieces of skin are transplanted between embryos of different breeds of fowl, the melanoblasts, by virtue of their migratory capacity, can leave the graft and supply an area of host down feathers with donor coloration (Willier and Rawles, 1940). However, under the conditions of this experiment the donor coloration is not permanently retained. During the replacement of the down by the juvenile and subsequent plumages, the donor color pattern "wears out," being superseded by that of the host. The present investigation was undertaken in an attempt to elucidate the mechanism responsible for this replacement.

It is well known that birds undergo periodic molts, the plumage which is shed being replaced by a process of feather regeneration. Each follicle gives rise to a succession of feather generations, each of which requires a new complement of melanophores. Moreover, the pigment cells have a high rate of turnover during the development of each individual feather. The pigment cells make their appearance in the epidermis at the base of the feather germ. Each melanophore occupies a fixed position within the primordia of the feather elements and discharges its pigment granules into the cells of the feather structure in which it is contained. This process of pigment deposition has been described in detail by Post (1894), Strong (1902), and Greite (1934). The melanophores are carried distally, as additional structures are formed at the base of the germ and, after completing their pigmentary function, are discarded. Melanophores are continually supplied at the growing base of the feather in order to provide pigment for each additional part of the feather as it differentiates.

The purpose of this investigation has been, therefore, to study the process by which a new complement of melanophores is made available for each successive feather generation. Is there a reservoir of stem cells at the base of the follicle, or does each new supply arise outside of the feather follicle? Is the process of providing the epidermis with melanoblasts from the dermis limited to relatively early stages of development, or does it continue throughout life? A number of studies on plumage have called attention to the occurrence of exceptional feathers possessing aberrant color patterns. These are particularly common in the hybrids from crosses between certain breeds. The usual explanation is that a change has taken place in the genetic composition of the melanophores. A solution to the problem of the mode of origin of the melanophores is therefore of considerable interest for the interpretation of pattern variation and replacement in successive generations from the same feather follicle.

II. STRUCTURE OF A REGENERATING FEATHER GERM

Since the feather germ is the site of the origin of the feather and its pigmentation, a knowledge of its morphology is necessary for an understanding of the observations and experiments which will follow. The histological structure of regenerating feathers was first described in detail by Davies (1889). Additional information has been provided by Lillie and his co-workers in a recent series of investigations dealing with the physiological mechanisms of feather development (see Lillie and Juhn, 1938; Lillie, 1940; and Lillie and Wang, 1941). The following description is based largely upon this later analytical study.

The growing portion of the feather is known as the feather germ and consists of an epidermal cylinder with a dermal core (Fig. 1). At its base there is a dermal papilla, which elaborates the vascular pulp. During most of the period of regeneration this papilla is encircled by the thickened epidermal collar which gives rise to the structural elements of the feather. The inner edge of the basal lip of the collar consists of a layer of "regeneration" cells, which lines the lateral surfaces of the papilla and is continuous with the wall of the follicle. When a feather is plucked during the course of regeneration, this layer of cells usually adheres to the papilla, the remainder of the collar and the pulp being torn away. The derivatives of the collar are (1) the cylinder cell layer which lines the surface of the pulp, (2) the intermediate cell layer, a close-set series of longitudinal ridges which comprise the primordia of the barbs and the shaft, and (3) a protective covering—the cornified sheath.

The apex of the feather is laid down in the early stages, and successively more basal regions are added by the continuous proliferation of the cells of the collar. In the young feather germ the barbs which will form the apex of the feather arise *in situ* in the epidermal covering as ridges which are perpendicular to the collar. After an initial period of growth the formative center of the shaft is established by the fusion of two adjacent barb bases occupying the so-called dorsal region of the collar circumference. The apices of additional barbs are laid down

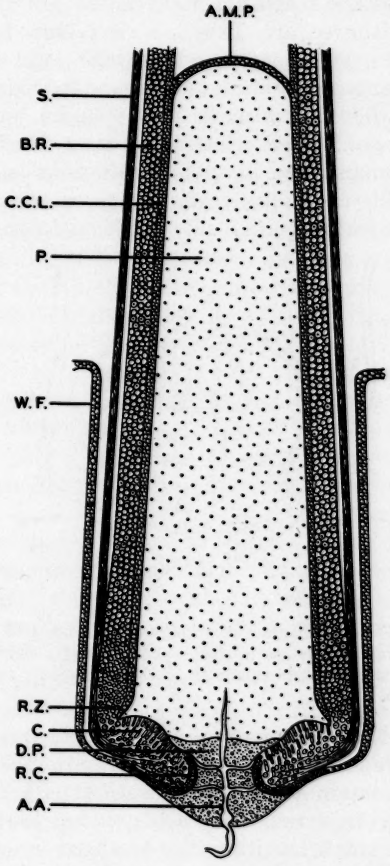

FIG. 1.—Diagram of a longitudinal section of a regenerating feather germ. *A.A.*=axial artery; *A.M.P.*=apical membrane of the pulp; *B.R.*=barb ridge; *C.*=collar; *C.C.L.*=cylinder cell layer; *D.P.*=dermal papilla; *P.*=pulp; *R.C.*=regeneration cells; *R.Z.*=ramogenous zone; *S.*=sheath; *W.F.*=wall of the follicle.

on both sides of a region opposite the shaft, known as the "ventral locus." Each successive apicobasal segment of a barb at the time of its formation occupies a successively more dorsal position in the collar. Thus, as each barb increases in length, its growing base is "tangentially displaced" around the collar until it fuses with the shaft upon its completion. These relations are most clearly demonstrated in split preparations (see Lillie and Juhn, 1938, Fig. 7), in which the feather germ is slit open longitudinal-

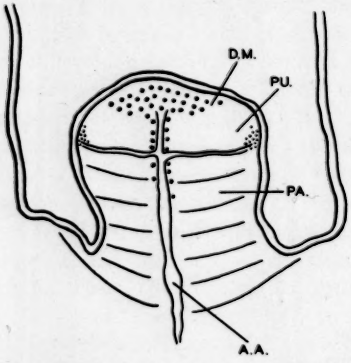

FIG. 2.—Diagram of a longitudinal section of a resting feather germ. *A.A.*=axial artery; *D.M.*= degenerate melanophores; *PA.*=papilla; *PU.*= pulp.

ly, the pulp removed, and the epidermal cylinder spread out flat. High-power examination of such preparations (Pl. I, Fig. 6) shows that each growing barb contains a longitudinally arranged series of melanophores, each melanophore being responsible for the pigmentation of the particular sector of the barb which it occupies. Hence the color pattern of the definitive feather, when projected back onto the feather germ, is seen to be a mosaic composed of numerous melanophores. The point of insertion of the growing bases of the barbs into the collar is known as the "ramogenous zone." The barbs become keratinized as they are carried away from the collar, and, after emerging from the mouth of the follicle, they break open the sheath at a point 1–2 cm. from the surface of the skin and spread out to form the feather vane.

The history of the pulp and its role in feather development has been elucidated by the work of Lillie (1940). The proliferation of the pulp by the dermal papilla keeps pace with the growth of the feather cylinder. The pulp is continually undergoing resorption at its apex, which occupies a position slightly proximal to the point of rupture of the sheath. At its apex the pulp is covered over with a special sheet of epidermal cells derived from the cylinder cell layer. This apical membrane of the pulp is responsible for the pulp-cap formation. At the conclusion of regeneration the pulp is almost entirely resorbed, and the collar is completely keratinized, forming the base of the calamus. The feather germ which remains at the base of the follicle enters a resting stage.

The resting feather germ (Fig. 2) is covered over with a single layer of epidermal cells, which is continuous with the wall of the follicle at its base. Its dermal component consists of the papilla and an apical cap of pulpy tissue.[2] In contrast to the description of Greite (1934) there is no basal thickening of the epidermis which corresponds to a collar. The collar becomes distinguishable gradually in conjunction with a general thickening of the epidermis, which usually takes place during the ninth to twenty-fourth hours of regeneration and does

[2] The term "resting papilla" has been used by Lillie and his co-workers with reference to the entire structure which remains at the base of the follicle after a completed feather has been plucked. In the present work it has been found convenient to confine the term "papilla" to the dense fibrous basal portion of the dermal component, thus distinguishing this portion from the apical-pulp residue and the epidermal covering. The entire structure is referred to as a "resting feather germ."

not acquire its characteristic structure until several days have passed. The apical-barb primordia make their appearance about the third day of regeneration, and the shaft primordium arises about the seventh day. Thus the typical morphology of the active feather germ is established, and the feather tip soon emerges from the follicle.

III. MATERIALS AND METHODS

The regenerating feathers of Barred Plymouth Rocks were chosen because of the relative intensity of the pigmentation process in this breed. Young hens, ranging in age from 3 or 4 months to over 1 year, were used as the source of the material. All feather germs were obtained from a single tract, the thigh region being selected as a convenient site.

1. An extensive histological study of the feather germ was made in order to ascertain the distribution and characteristics of differentiated pigment cells at all stages of regeneration. For the early stages completed feathers were plucked, and pieces of skin containing the regenerating feather germs were removed at the following intervals: at the time of plucking; at 3, 6, 9, 12, 18, 24, 36, and 48 hours after plucking; and at daily intervals thereafter up to the time of emergence from the follicle. For the later stages the length of the regenerated feather was taken as a more reliable criterion for obtaining comparable stages of development than time after plucking, owing to the variability in the onset, rapidity, and duration of the regenerative process. Following emergence (the time at which the feather tip first projects from the mouth of the follicle), feather germs were obtained at approximately 3–4-day intervals throughout regeneration. Any parts of the feather protruding from the follicle were cut off, and the superfluous skin surrounding the follicle was dissected away. The material was fixed with Allen's B-15 and Bouin's fluids, infiltration being carried out with butyl alcohol[3] and rubber paraffin being used for imbedding. Serial sections of the follicles were cut at 7 μ and stained lightly with Delafield's haematoxylin. At least two series (one transverse and one longitudinal) were made at each stage. These were supplemented with one or two additional series for several of the earlier pre-emergent stages. If a feather is plucked during the course of its regeneration, the feather germ does not enter a resting stage but immediately begins the formation of a new feather. Such feather germs were obtained at daily intervals up to 5 days after plucking, and similar series of sections were prepared for histological study.

2. An experimental analysis was undertaken in order to ascertain the distribution and properties of prospective melanophores (melanoblasts) during the process of regeneration. Feather germs were chosen at representative stages of development, and various components (e.g., the papilla, the collar, or portions of the pulp) were isolated by dissection and divided into pieces of a size suitable for grafting to young chick embryos. The tissue to be dissected was placed in a dish of sterile Ringer's solution kept at a temperature of 39° C., the dissection being carried out under a binocular microscope with the aid of very fine iridectomy scissors and watchmaker's forceps. In order to isolate the resting feather germ, a piece of skin containing a follicle from which the feather has been plucked is obtained. The follicle is cut open, and the surrounding tissues are removed from the feather germ which remains at the base

[3] The General Biological Supply House, Chicago, Illinois, suggested the use of butyl alcohol imbedding and kindly supplied me with the details of the technique.

of the follicle. Such an isolate measures about 1.0 mm. in length and approximately 0.5 mm. in diameter. In some experiments the entire feather germ was used as a graft; in others it was bisected longitudinally, and each half was divided into apical and basal portions. The active (regenerating) papilla, which is approximately the same size, was isolated and subdivided in the same manner.

For other components, actively regenerating feathers were plucked, and the completely keratinized distal portions cut off and discarded. The feather cylinder was then slit open by a longitudinal incision along the ventral raphe, and the pulp was carefully separated from its epidermal covering. Various regions of the pulp were isolated in the following manner. The apex of the pulp was cut off about 0.5 mm. below the tip and divided into four equal sectors. For the basal region a ring of tissue was excised from the periphery of the pulp at the level of the ramogenous zone. This was then divided up into pieces measuring about 0.5 mm. in each dimension. Similar pieces of the pulp periphery were excised from an intermediate region of the pulp at a level about halfway between the apex and the base. The epidermal apical membrane was stripped off the tip of the pulp with fine forceps and divided into squares measuring approximately 0.5 mm. along each side. Portions of the collar were obtained for grafting at different stages of regeneration. After removing the pulp from the feather cylinder, the collar was cut off at its junction with the ramogenous zone and divided by a transverse cut into two rings of equal width. These distal and basal strips were in turn subdivided into pieces of about the same size as that described for other components. Grafts were also obtained from feather germs of 2 and 5 days of age, after the preceding feather had been plucked in the middle of its regeneration. Such feather germs were isolated according to the procedure described for the resting feather germ. Owing to the high degree of keratinization of the epidermis under these conditions, it is possible to withdraw the dermal core from the epidermal covering. At 2 days the size of the feather germ has not increased appreciably, and only a small amount of pulp adheres to the apex of the papilla. The entire epidermal cap was divided into four equal sectors for grafting. At 5 days the feather germ has become considerably enlarged, and the pulp has elongated into a cone of tissue which can be isolated and tested separately. The basal region of the epidermal cylinder was cut off and again was divided into four equal sectors. In both cases the dermal papilla was subdivided according to the method previously described for other stages.

The isolates were tested for their melanophore-producing capacity (melanoblast content) by grafting to the coelom of 48–72-hour White Leghorn embryos (for details of this technique see Rawles, 1940). In order to eliminate any variability which might arise because of differences in phase of pigmentation, only regenerating feathers which were in the process of forming a black bar were used as experimental material. White Leghorns were selected as hosts because pigment cells are usually absent from the coelomic epithelium of this breed and, even when present, cannot possibly be mistaken for melanophores produced by the graft. Melanophores produced by grafts of portions of Barred Rock feather germs are large, expanded cells, occupying a circumscribed area, while those which are occasionally produced by the host are small, contracted cells, having a generalized distribution. A positive graft is characterized not only by the pro-

duction of melanophores but by their extensive migration from the graft into the surrounding host tissues. The number of melanophores produced and the degree of their migratory activity, while characteristic for each of the tissues tested, proved to be quite variable for certain types of grafts. An attempt has been made to provide a quantitative estimate of the relative capacity for melanophore production on the part of the different tissues tested. This can be done most directly by calculating the percentage of positive cases for each type of graft. However, this method deals only with the frequency with which the threshold level for melanophore production is attained and does not take into account variations in the number of melanophores produced by these positive grafts. A partial correction for this shortcoming can be made in the following way. Grafts which yield relatively few melanophores with limited migration have been recorded as producing traces. In calculating the percentages, these have been arbitrarily assigned one-half the value of a positive graft. Any other fraction of a comparable order of magnitude would serve equally well to give uniform adjustments. A certain amount of unmeasured variation remains.

Frequently the presence of the graft cannot be detected even after a careful examination of the body cavity. Undoubtedly, in some instances the situation of the graft is inadequate for proper nourishment and subsequent histogenesis. The graft may also become imbedded in the body wall or may be hidden in some obscure site. However, failure to recover the graft is unusual in the case of those tissues which consistently produce melanophores (e.g., the apex of the resting feather germ and the base of the pulp at early stages). The cases recorded as "no graft recovered" have therefore been included with the negative cases in computing the percentage of positive grafts. A small source of error is probably introduced by this procedure. The values obtained by this method of calculation are included in the tables of experimental results. It must be emphasized that these percentages have no significance as an index of the absolute melanophore-producing capacity of the respective tissues. Because of the sources of error previously mentioned and because of the small number of cases obtained for certain types of grafts, the figures can be considered only as rough approximations, which may, however, provide some basis for comparing the relative melanophore-producing capacity of the tissues which have been tested.

Certain isolates were also tested for their ability to furnish an area of host down feathers with donor coloration by grafting them to the base of the wing or limb bud of 60–72-hour White Leghorn embryos. The production of such a donor-colored area is dependent upon the presence in the graft of melanoblasts which can migrate into the skin of the host and penetrate the epidermal primordia of the down plumage (for details of this technique see Willier and Rawles, 1940).

IV. HISTOLOGICAL STUDY AND EXPERIMENTAL RESULTS

1. THE RESTING FEATHER GERM

The thin epidermal covering of the resting feather germ is entirely devoid of melanophores. However, the pulpy dermal apex is usually nearly filled with a dense blob of pigment, which, under high magnification, appears to consist of degenerate melanophores, each represented by a clump of large irregular granules (Pl. II, Fig. 14). Such aberrant granules may also be scattered around the periphery of the feather germ at the junction

of the papilla and the pulp, near the termination of small arterial branches. The main mass of pigment frequently extends down the axial artery of the feather germ (Fig. 2) and in one very extensively pigmented feather germ was found to continue into the external dermal tissue at the base of the follicle. Pigment is present only in the form of these clumps of atypical granules. Neither the feather germ itself nor the tissues of the skin surrounding it contain any active melanophores. The degenerate melanophores disappear rapidly after the onset of regeneration. There is no histological indication that these cells can renew their activity and thus serve as a source of melanophores for the next feather generation (see evidence in the following section). Since no visibly differentiated pigment cells are present, the melanophores which subsequently occupy the actively regenerating feather germ must arise from undifferentiated melanoblasts.

The following experiments demonstrate that the resting feather germ contains a supply of such cells (see Table 1). A number of grafts were made in which the entire resting feather germ was placed in the coelom of the White Leghorn host embryo. Because of the large size of the graft, this operation usually results in the death of the host. Two grafts of this type have been obtained. One was suspended in a strand of mesentery between the intestine and the body wall. A number of large, expanded melanophores had migrated out into this strip of tissue. The second graft became anchored to the body wall of the host embryo and underwent growth and morphogenesis during a 15-day sojourn in the host coelom. The size and degree of differentiation attained by this feather germ was comparable in every respect to that found in feather germs regenerating normally in their follicles for a comparable period of time. Furthermore, this feather germ was completely pigmented and possessed a barred pattern (see Pl. I, Fig. 7). The amount of differentiation obtained during a 15-day interval (as indicated by the number of bars produced) corresponds to approximately the apical one-fifth of a completed feather. Thus the resting feather germ of the Barred Plymouth Rock possesses a supply of melanoblasts capable of providing pigment for at least a considerable portion of the next feather generation.

TABLE 1

GRAFTS OF THE RESTING FEATHER GERM AND ITS PARTS

Pieces Tested	Site of Graft	No Graft Recovered	Production of Melanophores			Number of Embryos Examined	Percentage +
			+	Traces	−		
Entire germ	Coelom	0	2*	0	0	2	.....
Apical	Coelom	5	21	0	0	26	81
Basal	Coelom	21	3	3	6	33	13
Apical (exposed to acid for 2–3 minutes)	Coelom	11	0	0	5	16	0
Apical (exposed to acid for 15–20 seconds)	Coelom	13	0	4	3	20	10
Apical	Base of wing bud	8	3	0	0	11	.....

* One case of morphogenesis.

Information regarding the distribution of these melanoblasts was obtained by bisecting the resting feather germ longitudinally and dividing the halves into apical and basal portions. The capacity to produce migrating pigment cells was found to be localized for the most part in the apical pieces, each of the 21 grafts recovered being positive. These apical grafts typically yield a more extensive melanophore production than any of the other tissues tested (Pl. I, Fig. 10). The grafts of the basal portion were particularly difficult to locate, only 12 being obtained out of a total of 33 host embryos examined. That the failure to find these grafts is due primarily to their inability to form pigment cells is indicated by the much lower percentage of graft recovery for the apical pieces, when melanophore production has been inhibited by treatment with a solution containing salicylic acid. Of the 12 grafts obtained from basal pieces, only 6 produced any melanophores, and 3 of these produced only traces of pigment cells. The difference in melanoblast content between the apical and the basal pieces may be correlated with the morphological composition of the two parts. The epidermal covering appears to be a uniform and common constituent of the two types of grafts. However, the dermal component of the apical fragment consists primarily of pulpy tissue, while that of the basal piece is exclusively made up of the papilla and connective tissue of the adjoining derma. Hence it seems likely that the melanoblasts residing in the resting feather germ principally occupy the pulpy dermal apex. Their distribution is thus seen to coincide with that of the degenerate melanophores. Presumably, they represent different stages in the development of the same type of cell.

Additional support for the dermal localization of these melanoblasts has been obtained by treating the feather germs, prior to grafting, with a solution whose active component is salicylic acid (see Wang, 1941*a*).[4] Under the conditions of Wang's experiments it was possible to remove the ectoderm without excessive injury to the papilla. According to Wang, this is probably due to incomplete penetration, only the surface layers being appreciably affected. Because of their high melanoblast content only the apical pieces of resting feather germs were used in the present series of experiments. Immersion in the solution for 2–3 minutes resulted in complete inhibition of the grafts. Of 16 hosts examined, only 5 grafts were recovered, and these were entirely negative. Not only did the grafts fail to produce pigment cells, but in contrast to the untreated apical portions, which usually fray out rather loosely, the treated grafts retain their original form and have an opaque whitish appearance. Apparently, under the conditions of these experiments, this treatment results in the death of all the tissues of the graft. However, when the duration of exposure was reduced to 15–20 seconds, the inhibition, though still marked, was incomplete. Three of 7 grafts recovered were completely negative, as with longer treatment. The remaining 4 grafts, however, remained viable to some extent and produced a few migrating pigment cells. Apparently, with a less complete penetration of the injurious substance, a few more interiorly located cells are able to survive.

The question then arises as to whether these cells residing in the dermis can actually participate in the pigmentation of the epidermal structures. Their capacity

[4] I am indebted to Mr. Hsi Wang for suggesting the use of the salicylic acid solution, for information regarding the technique of its use, and for much stimulating discussion of the problems of feather pigmentation.

to do so was tested by grafting portions of the resting feather-germ apex to the wing bud of White Leghorn embryos. An area of black down feathers was produced in 3 embryos out of 11 examined. Two of these donor-colored regions were quite extensive, involving the proximal portion of the wing and the area surrounding its base; the third included only 8 or 10 feathers. This result demonstrates that the melanoblasts which occupy the pulpy dermal apex of the resting feather germ have retained their embryonic properties and are capable of extensive migration, of penetrating the epidermis, and of depositing pigment in the structural elements of the host down feathers.

The experiments on the resting feather germ help to explain the contrast between the black coloration of the down and the barred pattern of the definitive plumage of this breed. Montalenti (1934) studied variations in the barred pattern produced by feathers of different tracts. He concluded that the threshold to the formation of white bars is relatively high in more rapidly growing feathers, the width of the black phase increasing at the expense of the white. This interpretation was supported by the experiments described by Willier (1941), in which Barred Rock melanophores were transplanted into the more rapidly growing feather germs of White Leghorns. Watterson (1942) found that the down feathers of Barred Rock embryos have a very high growth rate and suggested that this might account for their uniform black coloration. The production of a barred pattern in the adult feather germ which underwent morphogenesis in the embryonic coelom (see Pl. I, Fig. 7) shows that the absence of barring in the down feathers is not due to extra-follicular factors. This result is in harmony with the conclusion of Willier (1941) that barring is produced by factors operating directly and independently in the individual feather germ. The result of introducing melanophores from adult Barred Rock feather germs into embryonic down feathers shows further that the properties of adult and embryonic melanophores differ in no essential respect. The difference in the coloration of the down and definitive plumages must therefore be due to different physiological conditions in the feather germs at different stages in their development, as indicated by the difference in their rates of growth.

2. FEATHER GERMS FROM PLUCKING TO THE TIME OF EMERGENCE

a) Previous feather completed when plucked.—Feather germs were examined histologically at short intervals during the first 2 days after plucking. An intensive and systematic search of each series, section by section, failed to reveal the presence of any active melanophores. During this period the degenerate melanophores gradually decrease in number. A few can still be found upon the third and fourth days but none thereafter. The aberrant granules are apparently dissolved or phagocytized and carried away. New active pigment cells make their appearance during the third day of regeneration. These cells are at first limited to the dermis and are faintly pigmented, containing sparse yellowish granules. They are typically elongate or spindle-shaped and are seldom branched (Pl. II, Fig. 11). There is no similarity whatever between these young active pigment cells and the clumps of aberrant granules, nor does the morphology of the degenerate melanophores ever diverge to the slightest degree in the direction of a form which could be considered a transition toward a state of renewed activity. As the active melanophores become more numerous, they are primarily restricted to the periphery of the dermis, and iden-

tical cells occasionally appear in the epidermis (Pl. II, Fig. 13). At the boundary between the dermis and the epidermis, pigment cells may be found which appear to lie partly in one tissue and partly in the other (Pl. II, Fig. 12). The morphology, distribution, and orientation of the melanophores in the 3–4-day feather germ (Fig. 3) is that which would be expected if they were migrating through the dermis and penetrating the epidermis, as Foulks (1942) observed. In this connection it is interesting to note that injection of Brown Leghorn capons with female hormone has no effect upon the color pattern of regenerating feathers if the period of hormone activity falls prior to the third or fourth day after plucking (see Juhn, Faulkner, and Gustavson, 1931). Willier (1942) has suggested that the response of melanophores to hormonal influences is conditioned, at least in part, by the properties of their epidermal environment. It is conceivable that the epidermis is an essential intermediary in the hormonal modification of the melanophores, so that the pigment cells cannot respond to hormone action until they have entered the epidermis. An alternative explanation is that hormones may be able to elicit a specific response only on the part of pigment cells which have attained a sufficiently advanced degree of differentiation and are actually engaged in melanin synthesis, regardless of the position which they occupy within the feather germ.

During the next 3–4 days the melanophores become more numerous and more extensively pigmented. The cells become densely packed with granules, which have undergone a progressive darkening through tan and brown to black. A similar series of transitions during the histogenesis of embryonic chick melanophores *in vitro* has been described by Hamilton (1940) from epidermal cultures and by Koller (1929) from cultures of mesodermal cells. At the same time there is a gradual increase in the number of melanophores occupying the epidermis where they soon begin their deposition of pigment. Throughout this period the melanophores occupying the dermis and the epidermis parallel one another in their degree of differentiation. On the basis of their morphology they are apparently identical in every respect, as

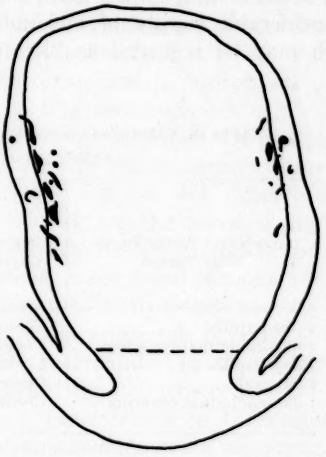

FIG. 3.—Camera lucida drawing of a longitudinal section through a 3-day feather germ showing the distribution of the melanophores. × *ca.* 60.

Strong (1917) concluded. The melanophores in the dermis aggregate at the periphery of the pulp, closely following the contours of the collar surface (Pl. II, Fig. 18). More distally they may be squeezed into the crevice between the bases of two adjacent barbs, as Strong (1917) previously observed. Under low powers of magnification the dermo-epidermal boundary seems to be quite coherent. However, when viewed under oil immersion it is seen that this boundary is not clearly defined in the region of the collar. This is particularly apparent at the level of the ramogenous zone, where the cells of the two tissues inter-

lace to some extent, as was noted by Lillie and Juhn (1938). Melanophores occupying the dermis adjacent to the collar can frequently be observed to insert a process into the epidermis (Pl. II, Figs. 15 and 16), demonstrating that the boundary between the two tissues provides no mechanical or physiological barrier to penetration by migrating cells.

b) Previous feather plucked in the middle of regeneration.—We have seen that the apex of the resting feather germ contains a considerable supply of melanoblasts, which may be regarded as "left over" from the previous regeneration and from which a considerable portion of the new feather's pigmentation can be derived. However, if the feather is plucked during the course of regeneration, the primordium of the new feather has no access to this supply. The early history of regeneration under these conditions is therefore of special interest. Following plucking, the exposed surface of the papilla is immediately covered over by epidermal regeneration cells. Careful histological examination failed to reveal the presence of any melanophores in such feather germs. As in the case of feather germs which began regeneration from the resting condition, new melanophores first appear about 3 days after plucking. However, the reoccupation of the feather germ by visibly differentiated melanophores is a much more gradual process, and the priority of the dermis is even more distinct. The keratinization of the outer layers of the epidermis is accelerated, and feather germs regenerating under these conditions are characterized by the presence of a cornified apical cap which persists until the rupture of a sheath. As a result of this process the feather-forming portion of the epiderdermis is thinner than usual, and the initiation of its pigmentation somewhat retarded during early stages.

TABLE 2

GRAFTS OF VARIOUS COMPONENTS OF EARLY FEATHER GERMS AFTER PLUCKING DURING THE PROCESS OF REGENERATION

Components Whose Pieces Were Tested	Site of Graft	No Graft Recovered	Production of Melanophores			Number of Embryos Examined	Percentage +
			+	Traces	−		
2-pay papilla	Coelom	1	0	2	8	11	10
2-day epidermal covering	Coelom	1	0	0	10	11	0
5-day papilla	Coelom	1	2	3	9	15	23
5-day pulp	Coelom	0	8	0	0	8	100
5-day epidermal covering	Coelom	2	1	4	0	7	60

The reoccupation of such feather germs by melanoblasts may be followed by the intra-coelomic grafting technique. The data from these experiments are recorded in Table 2. At 2 days after plucking, the melanophore-producing capacity of pieces of the papilla does not differ essentially from that found at other stages of regeneration (see Table 4), 2 out of 10 grafts producing a few pigment cells. The epidermal covering is devoid of melanoblasts at this stage, all 10 of the grafts obtained from pieces of the epidermal cap being negative. Apparently the renewal of the melanophore-producing capacity of the feather germs is in no way correlated with the overgrowth of the epidermis. At 5 days after plucking, the frequency of pigment-cell production

by grafts of parts of the papilla may be somewhat increased, 2 cases being positive and 3 producing traces of pigment cells out of a total of 14 grafts. Meanwhile, the pulp has acquired an abundant supply of melanoblasts, all 8 of the grafts of pieces of this tissue producing melanophores extensively. The melanoblast content of the epidermis at this stage could not be precisely determined, as it was impossible to be certain that this layer was completely separated from the adjacent pulp. However, an attempt to do so was made, and the grafts show that melanoblasts are not nearly so abundant in the epidermal layer as they are in the pulp. Of the 5 grafts recovered, only 1 produced very many melanophores, the remaining 4 grafts yielding only traces of pigment cells. This lag in the acquisition of pigment cells by the epidermis is probably responsible for the so-called "injury" effect described by Kuhn (1932). When feathers are plucked during the process of regeneration, the next feather generation frequently has a white tip.

3. POST-EMERGENT FEATHER GERMS

a) *The pulp.*—During the preceding stages active expanded melanophores may be found at all levels of the pulp. However, such cells are gradually restricted proximally, and shortly after emergence the concentration of these cells at the level of the ramogenous zone can easily be seen in preparations of the entire pulp, from which the epidermal structures have been carefully stripped away (Pl. I, Fig. 8).

At this time the melanophores in the pulp have a characteristic distribution with respect to the position of the pigment cells occupying the epidermis. When longitudinal sections are examined (Fig. 5, *B*), pigment cells in the epidermis are found only distal to the ramogenous zone, the collar containing practically no melanophores. The melanophores in the pulp are primarily localized in the region proximal to the ramogenous zone, the pulp above this level being relatively free of pigment cells. Thus the melanophores at the base of the feather germ appear to form a continuous series, the distal members residing in the epidermal barbs and the proximal members occupying the dermal tissue adjacent to the collar surface, a condition previously noted by Strong (1917). The precision with which this relation is established can be demonstrated most clearly in a series of cross-sections, examined in apicobasal order (Fig. 4). The melanophores in the pulp first appear opposite the shaft adjacent to that part of the epidermis where the barb ridges are not yet differentiated (ventral locus) and the degree of pigmentation is weak. As one progresses through successively more basal sections, the differentiation of barb ridges and melanophores in the epidermis is restricted toward the shaft, and at each level the distribution of the pigment cells occupying the pulp corresponds to the region of their disappearance in the adjoining epidermis. The melanophores in the pulp persist longest in the region next to the shaft, where the pigment cells within the epidermis have their greatest basal extent. This reciprocal correlation between the presence of pigment cells in the pulp and epidermis is a transitory condition, and the concentration of melanophores in the pulp at this level is steadily diminished as regeneration proceeds. These cells become contracted into spheres, densely packed with pigment granules, and are scattered around the periphery of the pulp as they are carried apically with its growth. Up until this time the tip of the pulp has remained free of pigment cells (Pl. I, Fig. 8). As regeneration continues, these cells gradually accumulate at the pulp apex and degener-

ate into clumps of atypical granules, as Foulks (1942) observed. A similar degenerative process has been described for pigment cells from cultures of embryonic chick mesoderm by Koller (1929). In this way a considerable mass of pigment is progressively built up (see Fig. 5), which will ultimately furnish the resting feather germ with its characteristic blob of degenerate pigment granules when the pulp is finally resorbed at the completion of regeneration.

Further information concerning the history of the melanophores in the pulp was provided by the following experiment. The apex of the pulp can be removed by clipping the feather near the mouth of the follicle. The wound heals rapidly, and regeneration proceeds as before. This procedure was applied to a series of 12 feathers in various stages of regeneration. After an interval of 2 weeks the feathers were plucked, and the pulp was removed and examined under the dissecting microscope. In the majority of these feathers the apical blob of pigment had been reconstituted, the size and density of the pigment mass approximating that of normal control feathers of the same age. However, in 2 of the feathers, which had been clipped when well on the way toward completion of regeneration and which were in the process of calamus formation when plucked, the apical accumulation of pigment was considerably more sparse. It appears that the pigment cells in the pulp, as well as those in the epidermis, have a constant turnover and are continually being supplied at the growing base of the feather germ and discarded distally. Apparently the rate of this process declines during the later stages of regeneration.

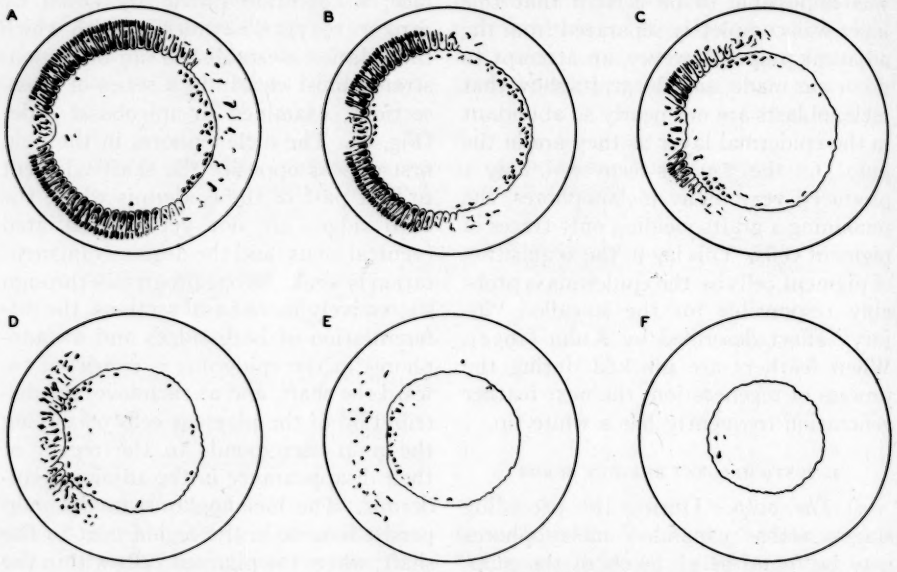

FIG. 4.—Camera lucida drawings of cross-sections through a feather germ shortly after emergence, showing the distribution of the pigment cells at successive apicobasal levels. × *ca.* 36.

Experiments in which the pulp has provided the material for grafting have revealed a close correlation between the disposition of the melanophores and the distribution of undifferentiated melano-

blasts, as shown in Table 3. In the early stages, shortly after emergence, while the tip of the pulp contains no melanophores as yet, pieces of the pulp apex have a negligible melanophore-producing capacity. Only 1 of 9 grafts examined showed a slight trace of pigment cells, the remainder being entirely negative. At the same stage, pieces of the peripheral portion of the basal pulp at the level of the ramogenous zone, carefully dissected free of epidermis, produce migrating pigment cells with a fairly high frequency, corresponding to the concentration of melanophores in this region revealed by the histological examination. Of 10 grafts examined, 5 produced fairly large numbers of pigment cells, and all the remaining 5 produced a few melanophores. Grafts of pieces of this basal region of the pulp at later stages show a decline in melanoblast content, corresponding to the decreasing concentration of melanophores in the dermis at this level described histologically. However, an appreciable melanophore-producing capacity is retained, 3 grafts being positive and 3 producing traces of pigment cells out of a total of 10 grafts. Although the tip of the pulp has little, if any, melanophore-producing capacity at early stages, its melanoblast content is markedly increased at later stages when it has accumulated an appreciable number of melanophores. Fifteen of 18 grafts recovered produced considerable numbers of migrating pigment cells (Pl. I, Fig. 9). That the capacity for melanophore production in pulp grafts is actually correlated with the regions of high melanophore content is shown by grafts of intermediate levels of the pulp in which the contracted melanophores are rather sparsely scattered. All 7 of the grafts which were obtained from this region were entirely negative.

Separate tests were made with pieces of the apical membrane of the pulp, and these yielded essentially negative results, only 2 out of 12 grafts showing traces of pigment cells. The epidermal component of the pulp apex is therefore of no importance in the production of new melanophores by grafts of this tissue. In the case of the resting feather

TABLE 3

GRAFTS OF VARIOUS REGIONS OF THE PULP OF POST-EMERGENT FEATHER GERMS

Regions Whose Pieces Were Tested	Site of Graft	No Graft Recovered	Production of Melanophores			Number of Embryos Examined	Percentage +
			+	Traces	−		
Apical, early stages (no pigment blob)	Coelom	4	0	1	8	13	4
Peripheral, at level of ramogenous zone, early stages	Coelom	1	5	5	0	11	68
Peripheral, at level of ramogenous zone, later stages	Coelom	2	3	3	4	12	37
Peripheral, at intermediate level, later stages	Coelom	4	0	0	7	11	0
Apical, later stages (with pigment blob)	Coelom	7	15	0	3	25	60
Epidermal apical membrane	Coelom	3	0	2	12	17	6
Apical, later stages exposed to acid for 2–3 minutes	Coelom	2	0	1*	8	8	0
Peripheral, at level of ramogenous zone, early stages	Base of limb bud	13	2	0	0	15	.....

* Shorter treatment (15–20 seconds).

germ the result of exposure to deleterious chemicals was offered as evidence for the dermal localization of its melanoblasts. The effect of this treatment upon melanoblasts whose dermal position has been clearly demonstrated—those of the pulp apex—offers an opportunity to justify this interpretation. Corresponding results were obtained. The melanophore-producing capacity of pieces of the pulp apex is completely inhibited with treatment for 2–3 minutes, each of the 8 grafts which were recovered being negative. In one case in which the time of exposure was shortened to 15–20 seconds a few melanoblasts survived and produced a trace of new pigment cells.

The melanophores which occupy the dermis and those which pigment the epidermal structures appear to be identical in the light of their similar morphology and correlated distribution. Additional evidence of their identity of origin is provided by the following observations. The great majority of the feathers of the F_1 hybrid from the cross of White Leghorn with Barred Plymouth Rock are white. Gross examination of the pulp of these feathers under the dissecting microscope reveals no indication of the presence of melanophores. However, exceptional feathers occur, possessing varying degress of black or barred pigmentation. A similar examination of such feathers reveals the presence of an accumulation of pigment cells at the pulp apex. In the case of the Barred Rock–Rhode Island Red hybrid, most of the feathers have black and white bars. The pulp apex of these feathers contains a blob of black pigment. Exceptional feathers also occur, having a coloration like that of the nonbarred parent and lacking the black pigment blob at the pulp apex. Likewise, when Barred Rock melanophores are transplanted to White Leghorn embryos, the pulp contains an apical blob of pigment, while donor-colored plumage is produced; but this also disappears when the host coloration replaces that of the donor. In the experiments of Wang (personal communication), when the feather papilla of a White Leghorn is transplanted to an empty follicle of a Barred Plymouth Rock, both the pulp and the epidermal structures acquire the pigmentation characteristic of the host. It is therefor apparent that a replacement of the melanophores which occupy the epidermis and provide the coloration of the feather is accompanied by a corresponding change in the pigmentation of the dermal pulp. This leads to the conclusion that the pigment cells of both tissues are derived from a common source.

Proof of the functional interchangeability of the melanophores residing in the dermis and the epidermis has been obtained by the following experiment. Pieces of the periphery of the basal pulp of early post-emergent feather germs—a tissue which has been shown by the intracoelomic method to possess a high melanoblast content—were grafted to the base of the limb bud of White Leghorn host embryos. The similar experiment with the resting feather-germ apex has shown that this is a much less favorable site for melanophore histogenesis. However, 2 out of 15 grafts of this type led to the production of a fairly extensive area of donor-colored down in the thigh and flank regions of the host, demonstrating that the melanoblasts which occupy the dermal pulp can take part in the pigmentation of the epidermal feather structures.

b) The basal region (papilla and collar).—A series of diagrams from longitudinal sections serves to illustrate the general trend in melanophore distribution at the base of the feather germ during successive stages of regeneration (Fig. 5). In the early stages pigment cells

proximal to the ramogenous zone are restricted to the pulp. The collar is almost entirely free of pigment cells, although numerous branched melanophores are present in the barb bases, where they undergo extensive multiplication (Pl. II, Fig. 17). At all stages the melanophores, when present in the pulp, have a greater basal extent than those of the adjacent phores finally become less numerous during the terminal stages of regeneration, disappearing entirely as the nonpigmented calamus is formed. The base of the collar always remains free of differentiated pigment cells. Although the appearance of the melanophores in the collar may vary somewhat with changes in phase, the formation of black and white

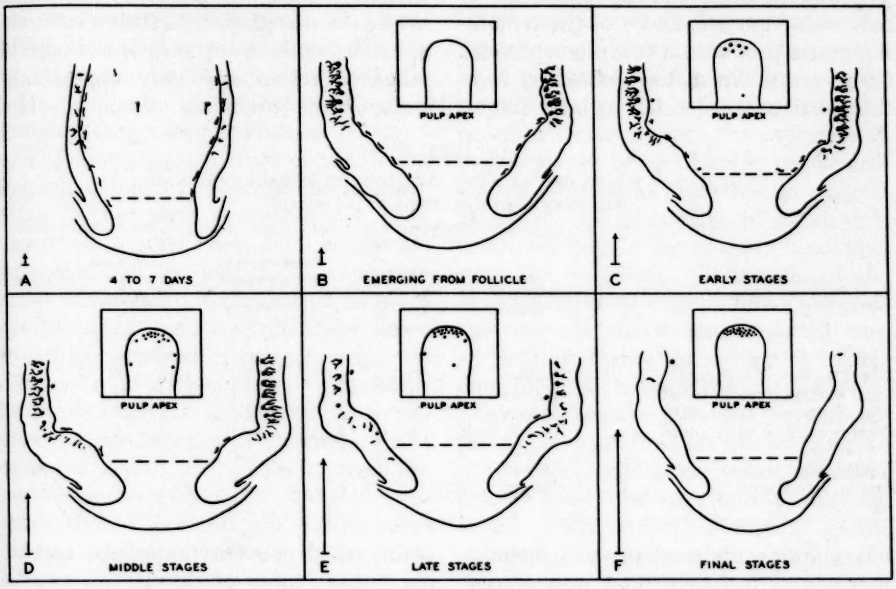

FIG. 5.—Camera lucida drawings of longitudinal sections through the base of feather germs at successive developmental stages, showing the distribution of the melanophores. The arrows are drawn to scale to indicate the relative length of the feather at each stage. × *ca.* 36.

epidermis. However, melanophores rarely appear within the dermal papilla itself. As regeneration proceeds, the melanophores in the basal region of the pulp become progressively less numerous, while faintly differentiated melanophores make their appearance in the collar. The melanophores in the collar gradually become more and more prominent and may come to occupy as much as the distal half of the collar. This condition is retained through the greater part of the period of regeneration. These melanobars does not affect the general trends in melanophore distribution.

The initial increase in the melanophore content of the collar might seem to indicate that it continues to receive melanoblasts from the dermis. However, experimental tests reveal that the concentration of melanophores is not a reliable criterion for estimating the melanoblast content of the collar, as shown in Table 4. Pieces of the distal part of the collar, at later stages of development when this tissue contains numerous pig-

ment cells, have a rather low capacity to produce new melanophores, traces of pigment cells being found in only 3 out of 7 grafts recovered. At early post-emergent stages pieces of the proximal portion of the collar, which contains no visibly differentiated melanophores, have a fairly good melanophore-producing capacity. Of a total of 18 grafts recovered, 5 produced considerable quantities of new melanophores, and 7 of the remaining grafts produced a few pigment cells. Of 10 grafts which were obtained from this portion of the collar at later stages, only 4 grafts produced any new melanophores, and 3 of these were only traces. Thus, as development proceeds, the basal portion of the collar undergoes a decline in its capacity to produce pigment cells, without, however, losing this property entirely. Its behavior in this respect parallels that of the peripheral pulp in the region of the ramogenous zone. Pieces of the basal collar, when grafted to the base of the limb bud, are capable of supplying melanophores to the host down feathers. Rather large areas of donor-colored down were produced in 2 cases out of 6 examined. Thus the grafting technique provides additional evidence of the similarity in the properties of those melanoblasts which occupy the collar and those which reside in the adjacent pulp.

We have seen that both the dermal and the epidermal components of the feather germ receive a continuous supply of melanoblasts at their base throughout the process of regeneration. The possibility that this steady supply of pigment cells might stem from a source in the dermal papilla was tested by means of intra-coelomic grafts. As shown in Table 4, extensive tests of pieces of the papilla failed to reveal any very appreciable melanophore-producing capacity. The pieces which were used as grafts consist not only of parts of the dermal papilla but also of portions of the layer of epidermal regeneration cells which adhere to the papilla when the feather is plucked. The frequency of positive grafts from this structure is uniformly low at all stages of regeneration (compare the base of the resting feather germ, Table 1). Pieces of the papilla of early post-emergent feather germs yielded only 2 positive grafts and 1 with a trace of pigment cells out of a total of 16 grafts obtained. At later stages of regeneration, 34 grafts recovered included only 5 positive cases and 2 with traces of melanophores. When these results are added to those obtained from the papillae of

TABLE 4

GRAFTS OF PIECES OF THE BASAL COMPONENTS OF REGENERATING FEATHER GERMS (PAPILLA AND COLLAR)

Components Whose Pieces Were Tested	Site of Graft	No Graft Recovered	Production of Melanophores			Number of Embryos Examined	Percentage +
			+	Traces	−		
Distal portion of late collar	Coelom	6	0	3	7	16	9
Basal portion of early collar	Coelom	3	5	7	6	21	40
Basal portion of late collar	Coelom	0	1	3	6	10	25
Basal portion of early collar	Base of limb bud	4	2	.....	.....	6	.....
Papilla, early stages	Coelom	4	2	1	13	20	13
Papilla, late stages	Coelom	16	5	2	27	50	12

5-day, 2-day, and resting feather germs, it is found that of a total of 129 embryos receiving grafts of this type, only 12 produced appreciable numbers of pigment cells. In view of these results, the presence of a permanent reservoir of melanophore stem cells in the basal components of the feather germ seems rather doubtful. The distribution of the degenerate pigment cells along the axial artery of the resting feather germ suggests the possibility that melanoblast stem cells are stored in the dermis outside the feather follicle and enter the feather germ by migration along vascular channels.

The histological picture of the development of pigmentation in the regenerating feather germ which has just been described may be interpreted on the assumption that the distribution and properties of differentiated pigment cells are acceptable as "markers" which may serve to define pathways of migration for undifferentiated melanoblasts. Admittedly, fixed material is inadequate for a conclusive demonstration of the migration of cells, particularly if the cells involved are not visibly differentiated at the time of this activity. However, the experimental analysis indicates that this assumption is generally applicable, and it provides the basis for an explanation which best accounts for the various data.

Dorris (1942, p. 105) observed that when pigment cells from pure cultures of neural-crest tissue are grafted to young chick embryos, melanophores appear in the host tissues external to the implant. Dorris concludes that "these differentiated cells from cultures still possess the capacity for growth and migration." The possibility that undifferentiated melanoblasts might have persisted in the cultures does not seem to be eliminated. Furthermore, the evidence from tissue culture indicates that, as a cell differentiates pigment, it loses its capacity to migrate and its activity is limited to the extension and retraction of processes (Hamilton, 1940). The melanophores which are most indicative of a penetration from the dermis into the epidermis—those of the 3-day feather germ—are very faintly pigmented. It may be that they are as yet insufficiently differentiated for the cessation of their migrating activity. On the other hand, undifferentiated melanoblasts may be penetrating so frequently that some are interrupted in the process by a premature differentiation of pigment. During the subsequent interval before emergence, only the processes of melanophores lying adjacent to the epidermis are seen to penetrate its surface. However, this demonstrated penetrability on the part of the epidermis, coupled with the peripheral distribution of the melanophores in the dermis, suggests that any available melanoblasts will continue to penetrate the epidermis. After emergence such direct evidence of penetration is lacking. Although the melanophores in the dermis are quite numerous at the basal region of early post-emergent feather germs and are almost exclusively found in the immediate vicinity of the epidermis, at no time after emergence do they appear to be penetrating the epidermal surface (compare Strong, 1915 and 1917). The distribution of the melanophores in the region of the ramogenous zone at early post-emergent stages suggests that the epidermis may have become impenetrable at this level, the melanophores aggregating against this barrier being confined to the dermis, while the adjoining epidermis remains relatively free of pigment.

The dermal component of the feather germ has a clear-cut priority in the acquisition of melanophore precursors at the beginning of regeneration. The subsequent acquisition of pigment cells by the epidermis appears to result from its

penetration by melanoblasts from the dermis. The provision of the epidermis with an adequate supply of melanophores is probably enhanced by the peripheral distribution of the pigment cells in the dermis. Probably purely physical factors, e.g., structural pathways, are responsible for this distribution, although a response to chemotactic stimuli is a distinct possibility. The factors which govern the distribution of the melanoblasts between the pulp and the collar are probably external to the cells themselves, which appear to possess uniform properties regardless of the age of the feather or the position which they occupy within it.

During the process of regeneration a continuous supply of melanoblasts is provided to the base of the feather germ. The available evidence gives no indication of the existence of a reservoir of stem cells within the feather germ and suggests that they are probably derived from some source within the dermal tissue external to the feather follicle. The history of the pigmentation of the pulp suggests that during the early stages most of these melanoblasts enter the epidermis so that the pulp apex remains free of pigment cells and their precursors. After emergence many melanoblasts fail to reach the epidermis and, after piling up at the ramogenous zone, are carried apically to the tip of the pulp. This behavior may be correlated with the demonstrated penetrability of the epidermis prior to emergence and the absence of such indications thereafter. If undifferentiated melanoblasts continue to enter the collar, they probably do so at a considerably diminishing rate. Furthermore, the experimental evidence indicates that a temporary reservoir of melanoblasts may be set up in the basal portion of the collar. This reservoir is depleted as regeneration proceeds and, if insufficient, might become exhausted. However, under normal conditions it seems likely that the proliferation of cells which enter the basal epidermis during the early stages of development may frequently be responsible for the major part of the pigmentation of the feather.

V. DISCUSSION

1. THE EXTRA EPIDERMAL ORIGIN OF THE MELANOPHORES

The evidence which has been accumulated in the present investigation offers cogent support to the theory that the melanophores responsible for the pigmentation of each new feather arise outside the epidermis. The observations and experiments on feather germs in early stages of regeneration show that melanoblasts and differentiated melanophores are present in the dermis before they can be detected in the epidermis. The histological observations have shown that penetration of the epidermis by melanophores occupying the dermis actually does occur. The intra-coelomic experiments testing the melanophore-producing capacity of various regions of the pulp have shown that the distribution of melanophores in the dermis is an index to the distribution of undifferentiated melanoblasts. The melanophores in the dermis aggregate at the periphery of the pulp adjacent to the epidermal surface and at the time of feather emergence have a precise distribution with relation to the pigment cells in the epidermis. At this stage, pigment cells are quite numerous in the basal region of the pulp, below the level at which melanophores appear in the epidermis, but occur only very sparsely distal to this level and are absent from the pulp apex. These pigment cells are morphologically identical with those which occupy the epidermis, and grafts to the base of the limb bud have demonstrated that the melanoblasts re-

siding in the dermal pulp actually can enter and pigment the feather structures of the host. All these data can be brought into agreement on the assumption that the feather epidermis acquires its pigment cells as a result of its penetration by melanoblasts from the dermis during early stages of regeneration. The process by which the epidermis receives its original supply of melanophores from the dermis during embryonic development thus finds its parallel in events which are continually recurring within the adult feather germ.

Additional support for this interpretation has been provided by Vilter (1935*a* and *b*), who worked with a black breed of domestic fowl. Following subcutaneous injection of thyroxin, feathers regenerating within the affected area have white tips. Histological examination of young feather germs developing under such treatment reveals that the epidermis lacks its usual supply of pigment cells, while a considerable number of the melanophores in the dermis have become crowded against the surface of the epidermis. According to Vilter, the thyroxin treatment delays the normal transfer of pigment cells from the dermis to the epidermis, thus leading to an absence of pigment at the feather apex.

Most of the previous studies on the pigmentation of regenerating feathers have concluded that the melanophores arise *in situ* in the epidermal structures. This interpretation has been based primarily upon the absence of sufficiently convincing indications that this was not the case, i.e., on purely negative evidence. The most accurate and extensive of these histological investigations was that of Strong (1915 and 1917), who studied the regenerating feather germs of Brown Leghorn and Barred Rock fowl. His observations included the peripheral distribution of the melanophores in the dermis, their morphological similarity to the pigment cells of the epidermis, and their greater basal extent. However, since the pre-emergent stages of regeneration were not studied, no evidence was found of an actual penetration of the epidermis on the part of melanophores occupying the pulp. It was therefore concluded that no relationship exists between the pigment cells of the two tissues. Champy (1935), in a study of the gonadal influences upon plumage, included some histological observations on the regenerating feather germs of a black breed of fowl. Several figures (4, 7, and 12) showing pigment cells in the collar and in the adjacent pulp are offered as a demonstration of the penetration of the epidermis by melanophores from the dermis. Much more extensive observations of a similar nature led Strong to the opposite conclusion.

Strong (1917, p. 103) pointed out that melanophores are not found in the pulp of the feather germs of several other species of birds. This is in line with similar observations on the feather germs of doves (Meirowsky, 1908), pigeons (Lloyd-Jones, 1915) and terns (Strong, 1902). However, dermal melanophores were found in the feather germs of doves by Post (1894) and Greite (1934). Undoubtedly differences in material are in part responsible for the differences in the conclusions reached by various workers. In the regenerating feather germs of Barred Plymouth Rocks the melanophores in the dermis are unusually numerous and become concentrated at the bases of the barbs and the apex of the pulp—regions at which they apparently meet a barrier to further migration. It is possible that the mechanisms of melanophore supply may differ in other species or breeds. On the other hand, with a less prolific melanophore production, all the available pigment cells may enter the

epidermis prior to their differentiation of pigment and hence fail to appear in the pulp in appreciable numbers.

2. THE EXTRA-FOLLICULAR ORIGIN OF THE MELANOPHORES

The presence of a reservoir of melanophore stem cells at the base of the feather follicle has been postulated by several investigators. This hypothesis follows logically from the acceptance of an epidermal origin of the melanophores. Greite (1934) described the resting feather germ as possessing a collar containing typical differentiated melanophores, which had been left over from the previous generation and could serve as a source of pigment cells for the next feather. This observation has not been confirmed by any other investigator. The resting feather germ is normally reactivated in the process of molting, and the previously completed feather may be retained in the follicle for some time after the initiation of regeneration. Thus, if the bird is nearing a molt, the feather germ at the base of a follicle which contains a completed feather may have terminated its resting condition and entered upon the process of a new regeneration. It seems probable that the resting feather germ described by Greite may fall in this category. Willier and Rawles (1940, p. 195) suggested that a reserve supply of melanoblasts might be stored in the layer of epidermal regeneration cells at the base of the follicle. In view of the present contention that the melanophores arise outside the epidermis, theories involving a reservoir of stem cells in this tissue must be discarded.

Champy (1935) proposed that a reserve supply of differentiated melanophores is permanently maintained in the dermal papilla. However, his Figures IV and IX do not clearly demonstrate the presence of active melanophores in the papilla of the young feather germ, and no evidence of their presence has been found by other investigators. Strong (1915) concluded on the basis of histological observations that the melanophores in the pulp arise in the dermal tissue outside the feather follicle. The experimental tests described in the present paper suggest that the papilla contains at the most a rather meager supply of melanoblasts as compared with other tissues of the feather germ. The collar and certain parts of the pulp are a much more consistent and abundant source of melanophores than are grafts of the papilla. It might be argued that the papilla is not a favorable site for melanophore proliferation and that it actually contains a more prolific supply of melanoblasts than these results would seem to indicate. Or it might be contended that the stem cells in the papilla are relatively indifferent and are determined to differentiate into pigment cells only after entering the pulp and the collar. It seems more probable that the melanoblasts enter the feather germ from the external dermal tissue, as suggested by the distribution of degenerate pigment cells along the axial artery of the papilla of the resting feather germ. The melanoblasts which migrate through the papilla might only occasionally be numerous enough to result in positive grafts.

This interpretation receives additional support from the fact that under certain experimental conditions the feather germ has been shown to receive its pigment cells from an extrinsic source. Rawles (1942) has shown that feather germs which have been deprived of their normal source of melanophores by the isolation of embryonic skin at a sufficiently early stage may become pigmented when the skin is grafted to the back of a pigmented host. Wang (1941*b*) has shown that, when adult feather germs are transplant-

ed between different breeds of fowl, the feather germ acquires the pigmentation of the host. In both these cases the transplanted feather germs acquire a new pigmentation as a result of their occupation by melanophores which have migrated in from the tissues of the host.

The present work provides no information as to the location of the melanophore stem cells within the dermal tissue external to the feather follicle. The experiments of Danforth and Foster (1929) show that, when skin is transplanted between newly hatched chicks of different breeds, the feathers regenerated by the graft always possess the coloration of the donor breed. Apparently during the course of embryonic development each separate area of the skin acquires a reservoir of melanophore stem cells capable of providing the feather germs which it contains with a permanent source of pigment cells.

3. BEARING ON THE REPLACEMENT PHENOMENON: DONOR-HOST COLOR MOSAICS

In contrast to the results of the skin transplantation experiments of Danforth and Foster (1929), Willier and Rawles (1940) have shown that the pigment cells which enter an area of host skin following embryonic grafting are unable to provide for a permanent donor coloration of the feathers. The present analysis furnishes evidence for a mechanism by which the transition in feather coloration from that of the donor to that of the host can be explained. The initial supply of melanophores received by the feather germ is exhausted, and a new supply migrates in from outside the feather germ, penetrates the collar, and replaces the pigment cells which have been used up. It is not necessary to postulate that some reaction on the part of the host feather germs is responsible for the elimination of donor pigment cells, since the evidence indicates that even normally developing feather germs do not contain a permanent reservoir of melanophore stem cells. This renewal of the melanophore content of the feather germ is not peculiar to the experimental conditions but continually recurs in normal feather regeneration, as each successive generation receives a fresh complement of melanophores. The present investigation provides no information regarding the failure of the donor pigment cells to set up a permanent source of precursors within the host skin. Recent experiments of Rawles (unpublished results) indicate that host-donor incompatibility is not involved and appear to verify the suggestion of Willier and Rawles (1940) that replacement is due to the introduction of an inadequate quantity of melanoblasts.

The replacement of donor melanophores by those of the host frequently leads to the formation of mosaic feathers, a variable amount of the apical portion of the feather being donor-colored and the remainder having the host coloration. Danforth and Foster (1929) and Danforth (1935, 1937, and 1939) have described similar mosaics which occasionally appear at the boundary of the graft when skin is transplanted between young chicks of different breeds. The two components of the mosaic generally occupy the apex and base, respectively, and are also due to melanophores of two different genotypes. However, in this case similar mosaics are usually produced by successive feather generations from the same follicle. The reproduction of similar apicobasal mosaics, consisting of areas pigmented by melanophores of two different genotypes, is very difficult to reconcile with any theory involving an epidermal origin of the melanophores. If we assume that melanoblasts are stored in the epidermal regeneration cells, the production of successive apicobasal mo-

saics would necessitate one of the following hypotheses. The melanoblasts of the two different genotypes might be arranged in a series of alternating rings at the base of the follicle. These would have to be indefinite in number, since no single ring would be a permanent source but would be used up and give way to the next. Each pair of rings would have to be of just the right magnitude to fulfil the melanophore requirements of a single feather in order to produce similar apicobasal mosaics in successive generations. Otherwise, we must suppose that there is a single permanent reservoir of mixed genotypes, one or the other of which is alternately favored or suppressed by the physiological conditions within the follicle. Danforth (1937) points out that, since successive mosaics often vary somewhat in pattern and may show an actual reversal in the position of the two components, there must be a certain latitude in the distribution of melanophores. An epidermal origin of the melanophores would not seem to provide a basis for this variability. The more probable explanation is that, when the initial melanoblast content of the feather germ is insufficient to provide pigment for the entire length of the feather, a new supply of melanoblasts may migrate into the feather germ and enter the collar. This replacement can occur at any stage in the development of the feather.

The form of the boundary between the donor- and host-colored areas in these graft mosaic feathers is of considerable interest. Although there is a certain amount of variation, the adherence to a general form is striking. In the majority of these mosaics the boundary between the two areas forms a V, with the apex at the shaft and the arms running parallel to the axes of the barbs. Frequently this transition is quite sharp, so that, of two adjacent barbs at this boundary, one is entirely donor-colored and the next entirely host-colored. (see Willier and Rawles, 1940, Pl. VI, Fig. 22; and Danforth, 1937, Fig. 1). This shows that the replacement of the initial supply of melanophores by a new complement does not occur simultaneously at all points in the collar but begins at a particular point, the ventral locus, with the formation of the apex of the first host-colored barb and progresses around the collar at about the same rate as the tangential displacement of the barb bases. The two vanes of the feather do not always behave as a unit and occasionally produce an asymmetrical pattern. However, the replacement of one color by the other independently in the two vanes conforms to the same pattern, the boundary paralleling the barb axes (see Willier and Rawles, 1940, Pl. VI, Fig. 23). Occasionally the transition is more gradual, and a certain amount of mottling may occur. However, even in these cases there is a marked tendency for the donor coloration to be replaced first at the tips of the barbs (ventral locus) and to persist longest in the region of the shaft, the V-shaped boundary simply being somewhat irregular and drawn out in a basal direction. The replacement of the melanophore content of the collar is thus seen to occur in a very regular and orderly fashion. The mechanism responsible for this regularity is a matter for speculation. The physiological properties of the collar are known to exhibit graded regional differences, as demonstrated by threshold of response to hormonal influences (Lillie and Juhn, 1932). It may be that the ventral region of the collar provides less favorable conditions for melanoblast proliferation and its supply is therefore used up more rapidly. It has previously been noted that differentiated melanophores, in both the dermal and the epidermal components of the feather germ, have a greater basal extent in the region of the shaft. It may be that this

region receives a more abundant supply of melanoblasts, owing perhaps to irregularities in the arrangement of vascular pathways. At any rate, it is apparent that the melanophore complement of the collar can be replaced during the middle of regeneration, and this replacement occurs in a definite sequence which results in a characteristic boundary between feather areas supplied by melanophores of different genotypes.

4. THE MELANOPHORE COMPOSITION OF EXCEPTIONAL FEATHER PATTERNS: HYBRID MOSAICS

Exceptional mosaic feathers sometimes arise spontaneously and provide interesting material for a verification of the interpretation presented and serve as a source of new information regarding the mechanisms involved. Barring is inherited as a sex-linked dominant. Birds whose genetic constitution includes a single barring gene (normal Barred Plymouth Rock females and the F_1 male offspring from interbreed crosses) generally produce a few exceptional nonbarred feathers. These have been described by several observers (Serebrovsky, 1925; Hertwig and Ritterhaus, 1929; Kuhn, 1932; Juhn, 1933; and Montalenti, 1934), all of whom agree as to the appearance of the aberrant feathers. The nonbarred coloration is black in the case of normal Barred Plymouth Rock hens and is similar to that of the nonbarred parent in the case of hybrid males. These atypical feathers may possess a uniform nonbarred coloration but are frequently mosaics, consisting of a barred tip and a nonbarred base. A similar situation has been observed in pigeons (Hollander and Cole, 1940), involving three dominant sex-linked genes which result in a lighter-colored plumage. Birds heterozygous for one of these factors have flecks of normal coloration. The shape and distribution of these flecks is rather irregular, and they are frequently quite small, although the size varies considerably.

The explanation generally offered for this phenomenon is that a genetic change has taken place in the melanophores occupying the atypical region. Serebrovsky (1925) suggested nondisjunction of the *X*-chromosomes, with the elimination of the one containing the barring gene. Hertwig and Ritterhaus (1929) proposed that only a part of the *X*-chromosome was lost. They also recognized the possibility of somatic mutation, a mechanism favored by Hollander and Cole (1940). Danforth (1937) pointed out that incomplete dominance (expressivity) might be responsible.

In contrast to these authors, Juhn (1933, p. 1266) has stated that a "genetic basis is embryologically untenable" for the explanation of aberrant feathers. This conclusion is probably based on the assumption that the melanophores arise *in situ* in the collar, whereupon the production of apicobasal mosaics would require the simultaneous mutation of all melanoblasts in the collar, possibly upon several different occasions in successive generations. Juhn proposed that physiological differences in the feather germ at different stages of morphogenesis (e.g., growth rate) are responsible for selecting one of the two potentialities present in the hybrid genotypes. In certain homozygous breeds of fowl, e.g., Brown Leghorns and Rhode Island Reds, two types of melanophores, one red and the other black, are normally present. One of these types may be restricted to certain sexes, to certain feather tracts, and also to certain areas of the feather, thus producing "natural" mosaics. The mechanism postulated by Juhn is probably responsible for determining the direction of the differentiation of melanophore precursors in these feathers. The type of melanophore occupying certain feather germs may be changed in the presence of female hormone, and there are definite threshold gradients around the collar

with respect to this transition (Lillie and Juhn, 1932). The replacement occurs most readily in the region of the shaft and frequently takes place almost simultaneously at all points in the collar. Accordingly, the boundary between the two successive areas is generally transverse to the shaft or may form a somewhat obtuse inverted V whose arms cut across the barbs instead of running parallel to them. The boundary between different-colored areas in "natural" mosaics is usually somewhat irregular but generally conforms to the same type produced by hormone injections.

In the hybrid mosaics, as well, one of the two areas (the barred portion) always occupies a particular part of the feather—its apical region—and there is a marked tendency for the aberrant feathers to be restricted to certain tracts. Possibly physiological differences in different follicles and at different times in the same follicle may favor one melanophore type more than another. However, there is considerable evidence that these physiological factors merely control to some extent the distribution of melanophores and are not responsible for the differentiation of two melanophore types. It seems more probable that the appearance of two types of pigment cells in the case of hybrid mosaics is due to a genetic change which takes place within the pigment cells themselves. Danforth (1937) noticed the similarity in the behavior of graft (donor-host) mosaics and hybrid mosaics with respect to successive feather generations and concluded that both are produced in the same way. In the case of pigeons, Cole and Finley (1941) have shown that X-ray treatment results in a marked increase in flecking, which may therefore be presumed to be due to a genetic change. In the fowl the similarity between hybrid and donor-host mosaics with reference to pigmentation patterns is quite striking. In both cases the two areas are separate and distinct, and the replacement of one by the other may occur asymmetrically with respect to the two vanes. Moreover, the boundary between the two component areas of the hybrid mosaics is frequently identical with that which characterizes graft (donor-host) mosaics, paralleling the axes of the barbs. This has been observed by the present author in large numbers of exceptional feathers from hybrids obtained by crossing Barred Plymouth Rocks with Rhode Island Reds and White Leghorns.[5] That this is a frequent occurrence in other material as well is shown by reference to the figures of previous investigators (see Serebrovsky, 1925, Figs. 2, 4, and 5; Kuhn, 1932, Fig. 43, *b;* and Montalenti, 1934, Fig. 74). Larger flecks in pigeons sometimes show the same pattern (Hollander and Cole, 1940, Fig. 2). The same pattern frequently occurs in connection with the injury effect (Kuhn, 1932, Fig. 48, *d*).

Most investigators of this problem have made observations on successive generations of feathers, and these are of considerable interest. Hertwig and Ritterhaus (1929) and Kuhn (1932) have stated that exceptional feathers are always replaced by exceptional feathers having an identical pattern. Montalenti (1934) also observed that only exceptional feathers were produced from follicles previously containing such feathers, but he found considerable pattern variation in successive generations. The same condition was found by Danforth (1937) for his graft mosaics, the pattern generally being similar in successive generations but seldom identical and occasionally very different (the reversal of apicobasal regions). Juhn (1933), on the other hand, studying hybrid mosaics in the F_1 male of the Brown Leghorn–Barred Plymouth

[5] The hybrid exceptional feathers were furnished for my examination through the kindness of Drs. B. H. Willier and N. T. Spratt, Jr.

Rock cross, has shown that this tendency for repetition is by no means constant. A large number of follicles were studied, normal as well as exceptional feathers being selected for observation. An analysis of the results indicates that the probability that a regenerating feather will be exceptional is about the same, regardless of whether or not the preceding generation was exceptional. When successive exceptional feathers were produced by the same follicle, in no case did the second-generation feather have a pattern identical with that which preceded it. Willier and Spratt (unpublished results) have observed that exceptional feathers may be replaced by normal feathers, in the case of normal Barred Plymouth Rock hens and also for the hybrid male of the Barred Rock–Rhode Island Red cross. Furthermore, Rawles (unpublished results) has observed that graft mosaics similar to those described by Danforth may be replaced by normal feathers.

On the basis of the reproduction of graft mosaics, Danforth (1937) concludes that a residuum of undifferentiated but predetermined chromatophores is carried over in the base of the follicle from one cycle to the next. However, the cases in which exceptional feathers are not reproduced would seem to weigh against this interpretation. This difficulty may be surmounted by the assumption that each new phase of the pattern results from the replacement of one source of precursors by another, the two separate sources being located in the dermal tissue external to the follicle. This mechanism allows all the leeway needed to explain the variations which may occur in successive feather patterns. The pigment cells seem to migrate along blood vessels, and the tendency toward repetition of pattern may find its explanation in the arrangement of the vascular channels.

Differences in the size of the aberrant area may be referred to the occurrence of genetic changes in melanoblasts at different stages in their processes of migration and multiplication. Those cases in which an entire feather or a large feather area possesses an atypical pattern suggest that the numerous melanophores which occupy the feather germ may be derived from very few primordial stem cells. Smaller areas would be due to changes arising relatively late in the process of pigment-cell distribution. The situation in pigeons (Hollander and Cole, 1940) shows considerable variation in the stage at which this change may occur, the size of the flecks varying considerably. Where the fleck is large, there is a marked tendency for repetition in successive generations; and the smaller the fleck, the less tendency for repetition. In most of the fowl hybrid mosaics the aberrant area is large and the tendency for repetition is prominent. However, in the case of the White Leghorn–Barred Plymouth Rock hybrid, many small flecks of black may occur, and there is no indication of a tendency for repetition.

The tendency for exceptional feathers to occur in groups has been observed by Serebrovsky (1925), Montalenti (1934), and in our own birds. Hollander and Cole (1940) describe the occurrence of chimerae in which a large area of feathers possesses the aberrant coloration which is permanently retained through successive molts. A similar case involving an area of black feathers in a Barred Rock hen has been observed in this laboratory. These are presumably due to genetic changes occurring at an early stage of development. The appearance of a small group of exceptional feathers in the adult bird indicates that these feathers have a common source of pigment cells. This offers strong support to the theory of the extra-follicular origin of the melano-

phores—a hypothesis which best seems to account for all the data which have been discussed.

VI. SUMMARY

1. The distribution of melanophores in the Barred Rock feather germ was studied histologically at all stages in the process of regeneration. The distribution of undifferentiated melanoblasts was studied by isolating various components of the feather germ at certain stages of development and testing their capacity to produce melanophores in intra-coelomic grafts to White Leghorn host embryos.

2. The resting feather germ contains no active pigment cells. Its pulpy dermal apex contains a blob of degenerate melanophores, which extends down the axial artery of the papilla and may reach to the external dermal tissue. These cells do not renew their activity. The dermal apex of the resting feather germ also contains a supply of undifferentiated melanoblasts, capable of providing pigment throughout at least 15 days of feather growth.

3. The feather germ which remains, after plucking during the course of regeneration, contains no melanophores and few melanoblasts. The dermal component of such feather germs has a clear-cut priority in the initial acquisition of new pigment cells.

4. New active pigment cells are first present in the dermis about 3 days after plucking and soon appear to be penetrating the epidermis. The melanophores occupying the dermis and the epidermis parallel one another in the degree of their differentiation and are morphologically identical. The melanophores in the dermis aggregate at the periphery of the pulp, and before the emergence of the feather they may insert processes into the epidermis.

5. Soon after the feather emerges, the melanophores in the pulp pile up in the region of the ramogenous zone, where they have a precise distribution with relation to the pigment cells in the epidermis. The melanophores in the pulp become prominent only just proximal to the region at which they appear in the epidermis. At this stage the pulp apex is free of pigment cells. As regeneration proceeds, the melanophores in the pulp become less numerous in the basal region and accumulate at the apex, where they degenerate to form the blob which characterizes the resting papilla when the pulp is resorbed at the end of regeneration. If the pulp apex is removed during regeneration, this blob of cells is soon reconstituted. Experimental tests show that the distribution of melanoblasts in the pulp coincides with that of differentiated melanophores.

6. Grafts to the base of the embryonic limb bud show that the melanoblasts occupying the dermis of the adult feather germ can enter the epidermis of the embryo and deposit pigment in the down feathers. Changes in the genetic constitution of the melanophores which occupy the feather epidermis are accompanied by similar changes in the type of pigment cell present in the pulp. These data provide convincing evidence that the occupation of the epidermis by pigment cells is the result of its penetration by migrating melanoblasts from the dermis during early stages of regeneration.

7. The basal part of the collar always remains free of differentiated pigment cells, although they become prominent in its distal portion as regeneration proceeds. Experimental tests show that a temporary reservoir of melanoblasts may be set up in the basal part of the collar. This supply becomes depleted during the course of regeneration. These cells can also furnish an area of host down feathers with donor coloration.

8. Melanophores rarely appear within the dermal papilla. Although a continuous supply of melanoblasts is furnished to the growing base of the feather germ, experimental tests give no evidence of the existence of a reservoir of stem cells within its basal components. It seems probable that these cells enter the feather germ from the external dermal tissue. This theory offers a plausible explanation for the replacement of melanophores of one genotype by those of another in the production of donor-host mosaics.

9. Evidence from the configuration of mosaic feather patterns is presented in support of the theory that the exceptional feathers of certain hybrids are produced in a similar fashion. Pigment cells of a different genotype are brought into the feather germ as its supply of melanophores is renewed.

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PLATE I

FIG. 6.—Split preparation in the region of the ventral locus, showing the longitudinal arrangement of melanophores in the barb ridges. ×100.

FIG. 7.—A feather germ which developed from the resting stage through 15 days of growth in the embryonic coelom. This graft was partly imbedded in the body wall of the host and, owing to its cramped quarters, became somewhat twisted and doubled back on itself. The sheath has been partly dissected away so as to reveal the barbs beneath. This feather germ was in the process of forming its second black bar, a portion of which can be seen at one end of the graft. ×12.

FIG. 8.—Ventral view of the pulp of a regenerating feather germ (soon after feather emergence) from which all the epidermal structures have been removed. Note the concentration of pigment cells at the base. ×12.

FIG. 9.—Pigment cells produced by an intracoelomic graft (*G*) of a piece of the apex of the pulp. Note the extent of migration. ×12.

FIG. 10.—Pigment cells produced by an intra-coelomic graft (the greatest concentration of pigment marks the site of the graft) of a piece of the apex of a resting feather germ. ×12.

PLATE II

FIG. 11.—Young melanophore in the dermis of a 3-day regenerating feather germ. ×970.

FIG. 12.—Young melanophore lying across the dermo-epidermal boundary of a 3-day regenerating feather germ. ×970.

FIG. 13.—Young melanophore in the epidermal layer of a 3-day regenerating feather germ. Note processes. ×970.

FIG. 14.—Degenerate melanophores in the apex of a resting feather germ. Note the large atypical granules, each clump representing a melanophore. ×450.

FIG. 15.—Melanophore in the dermis showing insertion of a process into the epidermis of a 7-day regenerating feather germ. ×970.

FIG. 16.—Melanophore in the dermis showing insertion of a process into the epidermis of a 5-day regenerating feather germ. ×970.

FIG. 17.—A dividing melanophore in the base of a barb ridge of a post-emergent feather germ. ×450.

FIG. 18.—Melanophore lying in the dermis along the surface of the collar of a young regenerating feather germ. ×450.

[6] I was not able to consult this work, which was cited by Biedermann (1928).

PLATE I

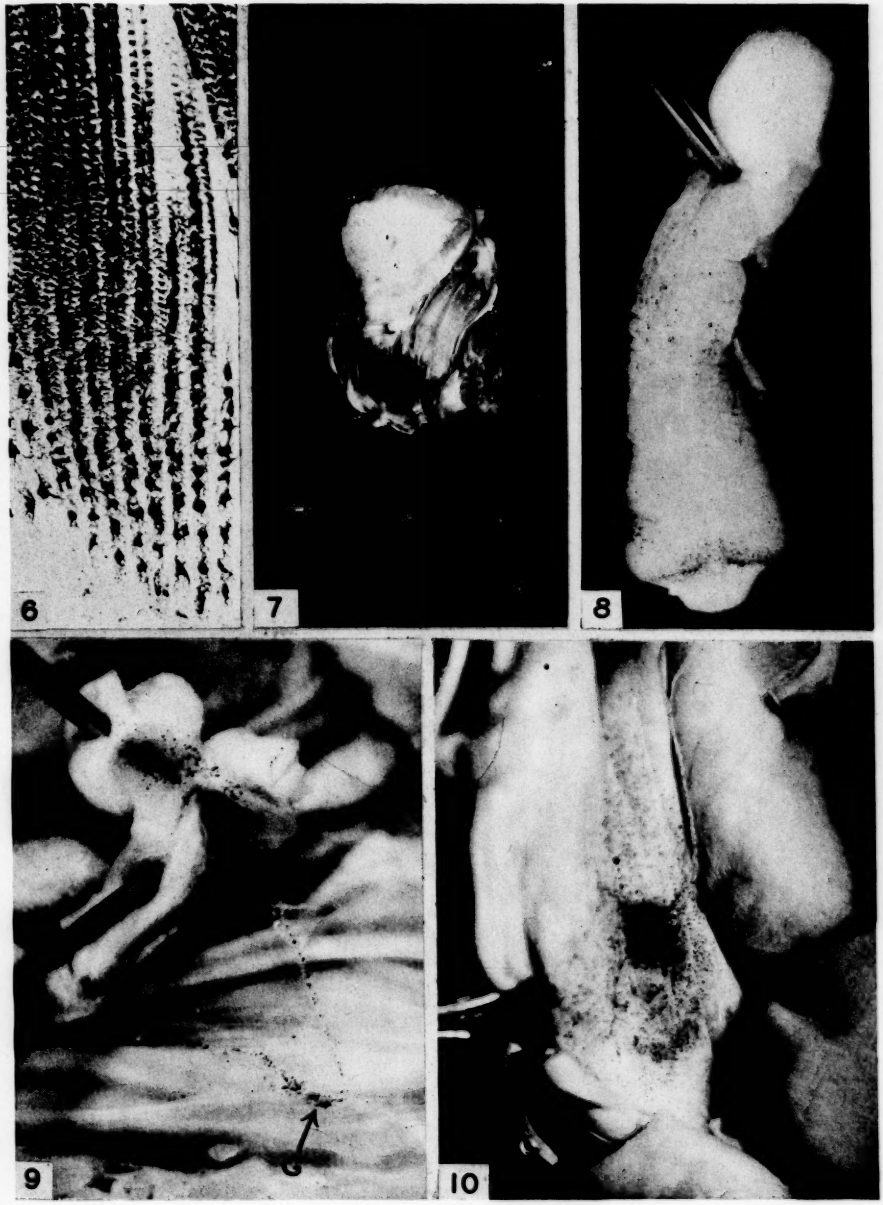
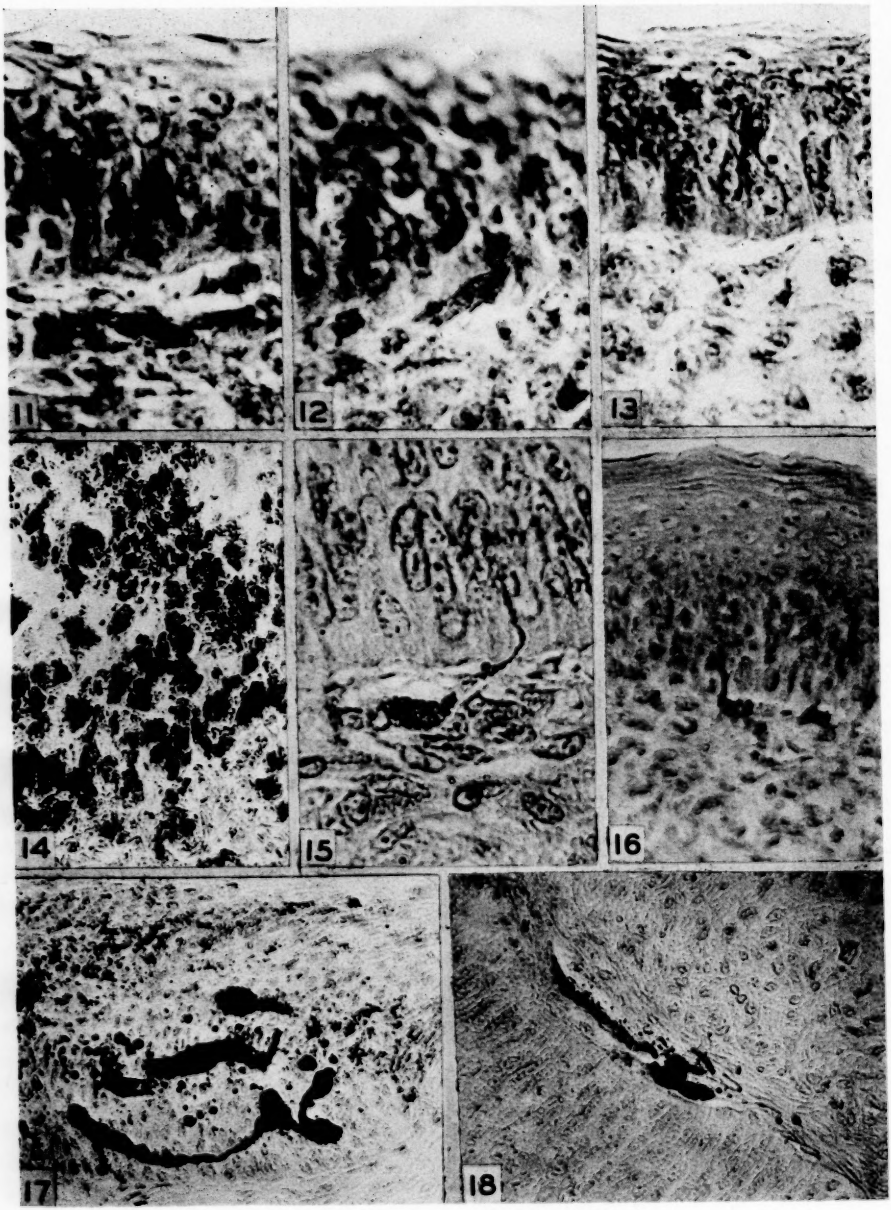


PLATE II



THE EFFECT OF DIFFERENT RELATIVE HUMIDITIES, DURING THE PUPAL STAGE, ON THE REPRODUCTIVE CAPACITY OF THE CYNTHIA MOTH, SAMIA WALKERI FELDER AND FELDER

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THE pupal stage of the Luna moth, *Tropaea luna*, is passed within a cocoon formed on the ground among the leaves of the larval food plant. In this situation it remains under relatively moist conditions throughout the winter. Ludwig (1942) exposed Luna pupae to different relative humidities and determined the effects of such exposure on the reproductive capacity of adults developing from the treated pupae. It was found that the cocoon is hygroscopic and tends to maintain a high humidity for the developing pupa. However, a high humidity is not required for the pupal development of this insect, since 85 per cent of those exposed to dry air produced adults. Some of these adults mated and deposited eggs, which hatched under favorable humidity conditions. Subjecting pupae to humidities between 56 and 100 per cent had no effect on subsequent oviposition; but, when they were subjected to lower humidities, there was a reduction in the number of eggs laid per female.

The cocoons of the Cynthia moth, *Samia walkeri*, are suspended from the branches of trees, where the pupae are subjected throughout the winter to the drying effects of the atmosphere. The present series of experiments was planned to determine whether Cynthia pupae are more resistant to desiccation than are those of the Luna moth, and whether there are differences in the hygroscopic properties of the cocoons of the two species. In these experiments adults were obtained from pupae kept at humidities ranging from 0 to 100 per cent, and it was possible to compare the oviposition of Cynthia moths with that of Luna moths whose pupae had been subjected to comparable humidity conditions.

Ludwig and Anderson (1942) found that the ability of eggs of saturniid moths to develop and hatch under adverse humidity conditions is associated with the size and shape of the egg and with its rate of development. It was shown that the rate of weight loss at comparable stages of development and under the same humidity conditions is determined by relative surface area; the smaller eggs dehydrate more rapidly than the larger ones. Also the flattened eggs of the Polyphemus moth, *Telea polyphemus*, dehydrate more rapidly than the more oval eggs of the Cecropia moth, *Platysamia cecropia*, although the eggs of these two species are of approximately the same size. Furthermore, eggs with a more rapid rate of development have a greater chance to survive at low humidities because they hatch before the vital limit of desiccation is reached. In the present experiments it was found that Cynthia eggs possessed a relatively low initial water content, and consequently a study was also made of the effect of the initial water content of the egg on the ability of the larva to develop and to hatch in dry environments.

MATERIAL AND METHODS

Cynthia cocoons were collected in and about New York City during January, 1942. No collections were made earlier in the season because it was found (Ludwig and Anderson, 1942) that exposure to cold is needed to break the winter dormancy of this species. The cocoons, with the contained pupae, were kept out of doors until needed for experimentation. All pupae were placed under experimental conditions before the beginning of warm weather in the spring. In the experiments the pupae were divided into two series, those retained in their cocoons until adult emergence and those removed from their cocoons. From 15 to 20 individuals were used in each series at each of the stated humidities. The pupae were placed in glass vials, weighed on a chainomatic balance, sensitive to 0.1 mg., and transferred to a humidifier at the desired humidity. Weight readings were made weekly until the approach of emergence, when they were made at intervals of several days, a reading usually being obtained within 1 day of emergence. The approach of the time of emergence was determined by noting the developing pigmentation of the wings and the brittlemess of the pupal covering as described by Ludwig (1942). When the moth was ready to emerge, the vial containing the pupa was removed from the humidifier and placed in a screen cage in the laboratory.

Empty cocoons were also placed in glass vials, weighed, and exposed to different humidities. They were then weighed daily until constant weight readings were obtained.

After emergence, female moths were placed each in a separate screen cage, where some were isolated and others were paired with males. Eggs of both mated and unmated females were collected daily and counted. The infertile eggs were used for the determination of water content and the fertile eggs for a study of the effect of different humidities on survival, on the rate of embryonic development, and on the weight and water content of the resulting larvae. The methods employed for making these determinations have been previously described (*op. cit.*). To determine whether low humidity affects the number of eggs produced or the mechanism of oviposition, each female was dissected after oviposition was completed, and the unlaid eggs were counted.

Constant relative humidities were obtained by the use of saturated solutions of various salts in the bases of ordinary desiccators. The approximate relative-humidity values of these solutions, listed in Table 1 (Ludwig and Anderson, 1942), were obtained by the use of a weighing hygrometer of the type described by Buxton (1931). A saturated atmosphere was obtained with distilled water and a dry atmosphere by the use of anhydrous $CaCl_2$. Throughout the experiment the humidifiers containing the pupae and eggs were kept in a Precision low-temperature control cabinet at a temperature of 25° C.

OBSERVATIONS

In Figure 1 are plotted the rates at which pupae lost weight when taken from out-of-door conditions and placed at different humidities. Each curve represents the average weight loss of all the pupae used in one series, excluding a few which were found to be parasitized or otherwise abnormal. For simplification, the chart is limited to results obtained at 0, 56, and 100 per cent humidities. The curves obtained for pupae kept at 16 and 76 per cent are similar in form to those shown and fall in their proper positions in respect to the latter. Pupae left in their cocoons

and placed in moist air appear to have gained weight during the first 3 days of exposure. This apparent increase in weight was due to the imbibition of water by the cocoon. In every series, weight loss was very gradual for a period of approximately 6 weeks. During this time the pupae apparently remained in winter dormancy. Throughout the dormant period the lower the humidity, the more rapid the rate of weight loss. Furthermore, during this period the cocoon seemed to retard water loss, since pupae left in their cocoons lost weight at a slower rate than naked pupae. At the humidities of 16, 56, and 100 per cent, the differences in rate of dehydration between the groups were found to be significant; differences as small as 1 per cent in rate of weight loss are statistically significant. However, at 0 and 76 per cent, they were not significant in all instances.

The period of gradual loss of weight was followed by one in which the loss occurred very rapidly. This change is characteristic of pupal metamorphosis, and the sudden downward slope of the dehydration curve marks the end of winter dormancy. During the period of active metamorphosis, weight loss occurred at approximately the same rate at humidities of 56 per cent and below, but more slowly at 76 and 100 per cent. At this time the cocoon did not appear to retard dehydration, since pupae left in their cocoons lost weight as rapidly as, and in some cases more rapidly than, naked pupae.

The changes in weight of empty Cynthia cocoons at different humidities were essentially similar to those plotted for the Luna moth (Ludwig, 1942, fig. 2). Cynthia cocoons lost weight at 0 and 16 per cent and gained weight at 56, 76, and 100 per cent humidities. In saturated air the cocoons continued imbibing water over a period of 10 days, and at equilibrium their weights were approximately 115 per cent of the original weights. Hence in this species also the cocoon serves to retain water and tends to retard atmospheric dehydration.

In Table 1 are listed the percentages of moths which emerged from pupae subjected to the various humidities. In obtaining these values, parasitized individuals were excluded. It is evident

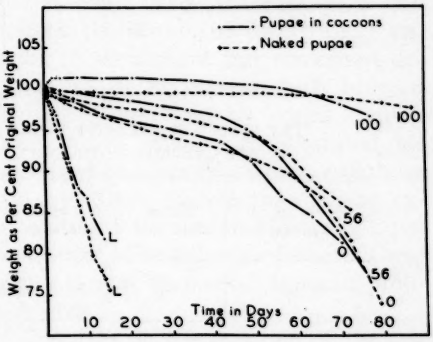

Fig. 1.—Rate of weight loss of Cynthia pupae at different relative humidities. Numbers indicate relative humidities. Graphs marked *L* show weight loss of Luna pupae at 0 per cent humidity.

from these figures that exposure to dry air increased mortality. However, this higher mortality at the lower humidities was due entirely to the failure of a number of moths to emerge, although they had completed development. Dry air also prolonged the time required for the completion of pupal development, this value at 0 per cent averaging 91 days and at the higher humidities only 77–81 days. These figures, however, do not give the time required for complete pupal development but only that portion which elapsed between the beginning of the experiment, when pupae were brought into the laboratory, until the adults emerged. The results indicate that dormancy was not influenced by

TABLE 1

EFFECT OF DIFFERENT RELATIVE HUMIDITIES ON THE PUPAE OF THE CYNTHIA MOTH

Approximate Humidity (Per Cent)	No. of Pupae	Per Cent Emerged	Time Required To Complete Development (Days)	Weight before Emergence, Per Cent Original Weight
100 (pupae in cocoons).....	12	83.3	80.7±1.45	95.3±0.55
100 (naked pupae).....	12	91.6	80.5±1.88	97.5±0.17
76 (pupae in cocoons).....	18	88.8	79.1±1.74	86.3±0.55
76 (naked pupae).....	14	92.8	78.8±2.31	84.1±0.63
56 (pupae in cocoons).....	17	88.2	77.7±1.63	77.0±0.94
56 (naked pupae).....	17	82.3	80.1±2.25	79.1±0.81
16 (pupae in cocoons).....	16	62.5	79.1±1.34	71.7±0.84
16 (naked pupae).....	14	85.7	78.7±3.13	72.5±0.88
0 (pupae in cocoons).....	21	33.3	91.1±3.29	69.0±1.97
0 (naked pupae).....	24	50.0	91.1±1.99	69.7±0.76

TABLE 2

THE EFFECT OF RELATIVE HUMIDITY ON THE NUMBER OF EGGS PRODUCED BY THE CYNTHIA MOTH AND ON THEIR WEIGHT AND WATER CONTENT

No. of Females	No. of Eggs Laid	No. of Unlaid Eggs	Total No. of Eggs	Weight of Eggs (Mg.)	Water Content of Eggs (Per Cent)
	Females Emerged at 100 Per Cent R.H.				
Mated 3....	222.7	85.3	308.0	1.84±0.005 (973)*	62.3±0.082 (246)
Unmated 6....	32.7	225.0	257.7		
	Females Emerged at 76 Per Cent R.H.				
Mated 3....	235.7	46.7	282.4	1.72±0.002 (2593)	62.3±0.030 (1759)
Unmated 9....	184.1	106.2	290.3		
	Females Emerged at 56 Per Cent R.H.				
Mated 2....	203.0	66.0	269.0	1.81±0.005 (1100)	61.2±0.082 (645)
Unmated 10....	64.0	215.1	276.1		
	Females Emerged at 16 Per Cent R.H.				
Mated 4....	192.3	54.2	246.5	1.75±0.005 (1256)	60.6±0.060 (593)
Unmated 10....	56.4	181.9	238.3		
	Females Emerged at 0 Per Cent R.H.				
Mated 2....	58.0	211.0	269.0	1.72±0.010 (111)	59.7±0.335 (34)
Unmated 4....	7.5	137.3	144.8		

* Figures in parentheses indicate the number of eggs weighed or dehydrated.

humidity but that development following dormancy was retarded in dry air.

In Table 2 are listed the data on egg production and oviposition of moths which completed pupal development at different humidities. In each case the data for mated and unmated females are listed separately. Mating of Cynthia moths is a definite stimulus for oviposition, since in each group the average number of eggs laid was greater for mated than for unmated moths. However, there were a few unmated moths which laid nearly all their eggs and a few mated moths which laid only a few eggs. The former group was composed largely of individuals which had completed development at the higher humidities, but the latter group included moths in each of the humidity series. The number of eggs deposited was definitely reduced when pupal development occurred in dry air. The number of eggs laid by mated moths which completed development at a particular humidity averaged 58 at 0 per cent, 192.3 at 16 per cent, and more than 200 at each of the higher humidities. However, the total number of eggs formed was not greatly reduced by low humidity, except in the case of three individuals, two of which during pupal development had been exposed to 0 per cent and the other exposed to 16 per cent humidity. These moths did not lay eggs, and on dissection relatively small numbers (45, 86, and 120, respectively) were found. These eggs were small and apparently incompletely formed. The inclusion of these figures, based upon abnormal cases, was responsible for the smaller averages obtained at the lower humidities. Thus the smaller number of eggs deposited by moths which had completed pupal development in dry air was caused by two factors. In some individuals the eggs failed to develop properly, and in others the eggs were apparently normal but only a few were deposited. Excessive dehydration thus appears to interfere with the mechanism of oviposition.

The figures listed in Table 2 on weight and water content include determinations made on eggs laid by both mated and unmated moths. Pupal development at low humidities apparently had no effect on the weights of the eggs laid, although, as previously mentioned, resulted in the production of small and incompletely developed eggs in a few individuals. From the figures there appears to be a slight but consistent decrease in water content with lowered humidity. Eggs laid by moths coming from pupae exposed to saturated air contained 62.3 per cent water, and those laid by others coming from pupae exposed to dry air contained only 59.7 per cent water. The difference between these means is more than seven times its probable error.

The data on the duration of the egg stage and on the weights and water content of larvae are essentially the same as those published by Ludwig (1942, Table 3) for the Luna moth and by Ludwig and Anderson (1942, Table V) for the Cynthia moth. Exposure of pupae to different relative humidities had no effect on the developmental capacity of eggs laid by the adults. Moths which emerged from pupae exposed to dry air mated and laid eggs which hatched as well at the different humidities as the eggs laid by moths whose pupae had been subjected to more favorable conditions. However, in the present series of experiments no Cynthia larvae were obtained from eggs kept at humidities below 16 per cent, although in the previous work 3.7 per cent of the eggs of this species hatched when kept at 25° C. and 10 per cent relative humidity.

DISCUSSION

It was observed that the cocoons of the Cynthia moth are hygroscopic and imbibe water when placed in a moist environment. The pupae, however, were found to lose weight at all humidities, including saturated air. These results are similar to those previously reported for Luna pupae and cocoons (Ludwig, 1942). But Cynthia pupae require a much longer time to complete their development when brought from out-of-door conditions and placed at 25° C. than those of the Luna moth. In the earlier work Luna adults were obtained in from 2 to 3 weeks after the pupae were exposed to 25° C.; while in the present series of experiments Cynthia moths did not emerge until the experiment had been in progress nearly 3 months. In spite of this prolonged exposure to experimental humidities, Cynthia pupae lost only a slightly greater percentage of their weight at comparable humidities than did Luna pupae. The former species thus possesses much greater powers to resist desiccation. Moreover, at low humidities Luna pupae lost weight at a uniform and rapid rate throughout the experiment, whereas Cynthia pupae lost weight very gradually during the first 6 weeks of the experiment and then more rapidly until emergence. It appears that the greater resistance to desiccation shown by Cynthia pupae is correlated with the persistence of winter dormancy for approximately 6 weeks after the pupae have been exposed to 25° C., while nearly all the Luna pupae used in the previous experiments were nondormant. A comparison of the rates of weight loss of Luna and Cynthia pupae at 0 per cent relative humidity is shown in Figure 1. During the first week of exposure at this humidity the average weight loss of the Cynthia pupae was 1.5 per cent of the original weight, while in the corresponding period the Luna pupae lost 11.1 per cent. Since the Cynthia pupae were dormant during this time, it was decided to select Luna pupae which were probably also dormant for a second comparison. During the first 2 weeks the average weight loss of Cynthia pupae at 0 per cent humidity was 3 per cent, and in the corresponding period and at the same humidity 5 dormant Luna pupae lost 8 per cent of their weight. Following the period of dormancy, the average weekly loss of Cynthia pupae in dry air was 2.7 per cent, as compared with 8.6 per cent in metamorphosing Luna pupae. Thus it appears that at corresponding physiological stages and at similar humidities Cynthia pupae are much more resistant to desiccation than are Luna pupae.

A comparison of the reproductive capacity of Cynthia and Luna moths whose pupae were subjected to the same humidities should be limited to the production of fertile eggs and to the ability of these eggs to develop under comparable environmental conditions. Cynthia moths were found to mate more readily in the laboratory than Luna moths. Furthermore, mating was found to be a definite stimulus to oviposition in Cynthia but not in Luna. Ludwig (1942) found that when Luna pupae lost from 15 to 17.4 per cent of their original weight there was a definite reduction in the number of eggs laid by moths which developed from such pupae. A weight loss of 17.4 per cent occurred when naked pupae were subjected to 56 per cent humidity, and egg production decreased from an average of more than 180 per female at each of the higher humidities to 123.3. In the present experiments no great reduction in the number of fertile eggs was observed except in moths which emerged from pupae exposed to dry air, in which the

weight loss during pupal development amounted to approximately 30 per cent. Hence it appears not only that Cynthia pupae are more resistant to desiccation than Luna pupae, but that a greater amount of dehydration must occur before there is a decrease in the egg production of moths which develop from the dehydrated pupae. Cynthia pupae are therefore better adapted to survive at low humidities than are the pupae of the Luna moth.

Ludwig (1942) obtained a high percentage (26.3–45.8 per cent) of hatching when Luna eggs were subjected to dry air at 25° C.; whereas Ludwig and Anderson (1942, Table V) found that at the same temperature Cynthia eggs did not hatch below 10 per cent humidity, and in the present experiments no larvae were obtained at humidities below 16 per cent. This difference in the mortality of the eggs of the two species at low humidities may be correlated with their initial water content. Newly laid Luna eggs were found to have an average water content of from 66.6 to 67.8 (mean, 67.4 ± 0.008) per cent; whereas, as Table 2 shows, these values for Cynthia eggs ranged from 59.7 to 62.3 (mean, 61.9 ± 0.028) per cent. It seems likely that, because of their low initial water content, Cynthia eggs exposed to low humidities become desiccated beyond the vital limit before the larvae are ready to hatch.

SUMMARY

1. Cynthia pupae were found to lose weight at all humidities, including saturated air. This weight loss was very gradual for a period of about 6 weeks, during which the pupae remained in winter dormancy. This period was followed by another which extended until adult emergence, during which weight loss was very rapid, particularly at the low humidities.

2. Cynthia cocoons are hygroscopic and tend to retard the loss of water from the pupae during the period of dormancy. However, during active metamorphosis, the cocoon did not appear to retard dehydration, since pupae inclosed in their cocoons lost weight as rapidly as naked pupae.

3. Cynthia pupae, in comparable physiological stages and under the same humidity conditions, are more resistant to desiccation than Luna pupae. Furthermore, in the former species the pupae can be desiccated to a greater degree before there is a decrease in the egg production of moths which develop from the dehydrated pupae. They are thus better adapted to overwintering at low humidities than are the pupae of the Luna moth.

4. The reduction in the number of eggs deposited by moths which emerged from pupae kept at low humidities was caused by two factors. In some individuals the eggs failed to mature fully. In others they were apparently normal, but only a few were deposited. Excessive dehydration thus appears to interfere with the mechanism of oviposition.

5. The ability of the eggs of saturniid moths to develop and hatch at low humidities appears to be correlated with their initial water content. Thus Luna eggs were found to contain an average of 66.6–67.8 per cent water, and a high percentage of them hatched when placed in dry air; whereas Cynthia eggs contain an average of from 59.7 to 62.3 per cent water, and in the present experiments they did not hatch at humidities below 16 per cent. Evidently at the lower humidities they became dehydrated beyond the vital limit before the larvae were ready to hatch.

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FURTHER STUDY OF THE ACTION OF DRUGS ON THE HEART OF THE COMPOUND ASCIDIAN, PEROPHORA VIRIDIS

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NUMEROUS studies on both invertebrate and vertebrate hearts have demonstrated that some kind of neurohumoral control exists for the heart beat. Similar studies on the ascidian heart, in which periodic and rhythmical reversal of the direction of beat in the tubular organ occurs normally, have yielded certain apparently conflicting and in some cases even negative results. In the case of the compound ascidian, *Perophora viridis*, drugs quickly penetrate the tissues, even when the siphons are closed, as shown by the immediate reaction of the heart. The effects produced by the drugs are different from those caused by mere transfer of the animal from one dish of sea water to another. In a preceding study (Waterman, 1942) the reactions of the animal were examined following exposure to acetylcholine, mecholyl, adrenalin, atropine, and colchicine. When the results were interpreted in the light of the evidence secured by others on different invertebrate hearts, it was concluded that a cholinergic type of neurohumoral system of heart action exists in *Perophora* and the heart is neurogenic.

The drugs acted first on the pacemaker mechanism (centers of automatic stimulation at each end of the heart) and later on the contractile mechanism; the two competing pacemaker mechanisms were differentially susceptible; certain of the drugs increased the duration and number of beats of the abvisceral phase as well as the extent of variability, i.e., acetylcholine, mecholyl, and adrenalin; and the relative dominance of the abvisceral phase over the advisceral was strengthened. With frequent changes of the experimental solution, adrenalin caused the heart of some animals to beat in one direction for hours, when normally the length of time involved is but a few minutes. Otherwise the animals exhibited few effects other than variable degrees or length of contraction and retarded or inhibited blood circulation in high concentrations. The ascidian heart reacts, at least to some of the drugs tested, more like certain crustacean hearts than like either molluskan or vertebrate hearts, which are myogenic but innervated (Prosser, 1942).

At the request of Dr. Prosser the original data of these earlier experiments were sent to him for calculation of the heart rates. He found that in each of six

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experiments with acetylcholine and in two with mecholyl (for example) a smooth, marked acceleration in rate occurred. Acceleration occurred in both pacemakers, but the threshold for the abvisceral beat was lower than that for the advisceral beat. In one typical experiment his calculations showed that the frequency in beats per minute for successive abvisceral phases each lasting $1\frac{1}{2}$–2 minutes was as follows: in sea water 56.7, 55.2, 55.6; then in acetylcholine (10^{-5}) 50.8, 64.3, 64.1, 62.1, 66.6, 65.3, 66.8 (Prosser, *loc. cit.*).

This preliminary study has been continued by exposing whole animals to a larger number of the drugs which have been employed by others in pharmacological studies on the heart, i.e., eserine, nicotine, pilocarpine, pilocarpine preceded by atropine, and acetylcholine preceded by either eserine or atropine. The effects of nicotine on the ascidian heart have been studied by Schultze (1901), Hunter (1903), and Kruckenburg (quoted by Hunter), working with *Molgula* and the *Salpae*. Bacq (1935*a*, *b*, *c*; 1939) has studied the effects of acetylcholine and eserine on ascidians and has analyzed ascidian tissues for the presence of acetylcholine and other substances.

MATERIALS AND METHODS

Perophora viridis is a small, transparent, green-colored, compound ascidian in which the ascidiozoöids are connected by stolons. Reversal of the direction of the heart beat in ascidians is a well-known phenomenon said to result from the alternating dominance of pacemaker mechanisms, or centers of automatic stimulation. These are located at each end of the tubular-shaped heart and function periodically and rhythmically. The cause and mechanism of reversal are poorly understood; in fact, there have been few physiological studies made on ascidians. In most ascidians which have been studied it has been found that one of these centers is more powerful than the other—at least the number of beats in one direction is larger than in the other. The center which is dominant at a given moment is the one possessing the greatest frequency. Beating is reversed when the chronotropism of the resting center becomes superior to that of the functioning one. Recent studies seem to agree that reversal is due to a difference in activity of the two centers and not to the "back-pressure" assumption of some of the earlier workers. Of all the drugs tested thus far, only adrenalin has provoked continuous beating in one direction for any great length of time, but other drugs will lengthen the abvisceral phase and shorten the advisceral. Further investigation of this phenomenon is needed.

According to Skramlik (1930), ascidians possess a certain degree of ability to change the direction of the circulation of blood in the body. But there is still considerable uncertainty concerning the nervous organization within the heart. This problem is being attacked in *Molgula manhattensis*, which is more favorable material, since the small size of *Perophora* is a barrier to such a study. Furthermore, the present interest in drug action has raised the question of the reaction of *Perophora* to natural environmental changes, i.e., temperature and salt concentrations. Generally, littoral ascidians are found attached to algae, stones, and wharf piles in locations protected against much wave action and yet exposed to an ever changing supply of fresh sea water.

In *Perophora* the length of the abvisceral phase (toward the branchial basket and stolon) is generally slightly longer in time and number of beats than that of the advisceral (toward the viscera). This is shown in the text figures. However,

the number of beats in both phases is highly variable both in the same and in different individuals (Table 1). Pulsations originate from centers at either end of the heart and pass as a succession of wavelike constrictions along the tube toward the opposite end. Rate and amplitude of contraction are commonly quite uniform throughout either phase but may decrease slightly at the beginning and end of each reversal period, when the direction of blood flow changes. Reversal is not an abrupt phenomenon, i.e., dominance of the opposite heart center may involve as much as several seconds in overcoming the active center.

Stock solutions of nicotine, eserine, atropine, acetylcholine, and pilocarpine were made at daily intervals and appropriately diluted in sea water just previous to their use. Before the animals were transferred to the experimental solutions, the condition and rate of heart beat in each normal quiescent animal in sea water were determined, as well as the length of the rest periods and the extent of variability in the number of beats in both phases. The lowest and highest number of beats (see text figures for specific examples) recorded for each phase during the period of observation are listed in Table 1. These records, together with counts made when the animals were merely transferred from one dish of sea water to another, served as controls for the action of the drugs; it was necessary to establish a norm for each animal studied. All experiments were performed at room temperature, which varied from day to day between 22° and 29° C. Indicators of the effects produced upon heart activity included change in number of beats in both phases, amplitude of beats, change in normal variability between successive counts, length of the two phases as measured by a stop watch, length of the interval between reversals, and recovery capacity in the experimental solution and in sea water. The amplitude of contraction could be estimated optically only.

The studies reported in this paper were made at Woods Hole, Massachusetts, during July and August, 1940, 1941, and 1942. Since different ascidian hearts are so variable in their action, it was deemed necessary to repeat each original experiment at least twice. In spite of this variability, the data obtained fall into definite catagories peculiar to the different drugs. Limitation of space does not permit the detailed description of every case, nor is this necessary. One example considered typical for the effective concentrations of each drug is discussed, and text figures of seven cases are included, as well as a table which summarizes certain information. The numerous exploratory experiments are not formally considered. At times certain generalizations are made from recorded data, which for lack of space are not listed herein, i.e., the effect of daily repeated doses of nicotine on the same animal.

In presenting the data in graphic form, it was found necessary in the case of each ascidiozoöid to separate the advisceral phase counts from those of the abvisceral and to construct two graphs for each animal, one representing consecutive advisceral counts (circles) and the other the abvisceral (triangles). However, it must be borne in mind that these two phases alternate with each other. For example, in any of the text figures each point on either graph represents the number of beats in the particular phase and its length in time from the end of the previous phase. The length of time which has elapsed since the beginning of the experiment can be estimated by adding the time values of any two corresponding points in both graphs plus the time values represented by any letters which may

TABLE 1

EFFECT OF VARIOUS DRUGS ON THE ACTION OF THE HEART OF *Perophora viridis*

Case	High and Low Counts of Normal				High and Low Counts of Experimental						Size of Animal	Room Temperature (°C.)	Comments
	Abvisceral Phase		Advisceral Phase		Concentration of Drug	Minutes Studied	Abvisceral Phase		Advisceral Phase				
	Number of Beats	Time in Minutes	Number of Beats	Time in Minutes			Number of Beats	Time in Minutes	Number of Beats	Time in Minutes			
B.....	148 106	1:40 1:19	127 79	1:30 1:56	Eserine 1:200,000	55	310 2	3:28	127 2	1:39	Large	28	Increase in some abvisceral counts for first 25 minutes; advisceral unaffected at first, then great irregularity in both phases, especially advisceral
A.....	197 161	3:10 2:30	118 99	1:52 1:34	Eserine 1:5,000	360	160 5	2:29	9 1	1:18	Medium	28	Beating reduced to ends of heart after 10 minutes; recovered after 4½ hours in sea water
D.....	173 149	2:15 1:56	141 122	1:58 1:46	Eserine 1:250,000	120	212 121	2:50 1:45	133 66	1:57 1:59	Large	25	Some increase in abvisceral counts at first and depression of advisceral number (see Fig. 3)
B.....	142 121	2:25 1:59	124 97	1:58 1:36	Nicotine 1:500,000	57	329 92	7:15 2:15	145 2	2:15	Medium	22	Temporary pauses of 5–35 seconds in length; time between reversals of 10–25 seconds; abvisceral count increased; irregularity intensified
A.....	140 117	1:56 1:35	124 105	1:46 1:28	Nicotine 1:50,000	25	133 7	1:40	104 1	1:17	Medium	26	Only one high count of 282 beats lasting about 8 minutes; quickly depressive, faster in advisceral phase
C.....	203 20	2:15 2:16	144 12	1:51 1:13	Pilocarpine 1:1,000	85	275 20	3:14 3:15	133 1	1:36	Medium	24	Great variability in counts of normal heart; beating reduced to vacillations after 25 minutes of exposure
E.....	152 129	2:02 1:44	164 114	2:14 1:34	Pilocarpine 1:5,000	77	356 15	4:30 4:13	98 16	1:23 1:15	Medium	23	Immediate stimulation of abvisceral phase followed by progressive decrease in counts; little variation

intervene between them and the first points on the graphs. Thus in Figure 1 letter *A* marks the point at which a single ascidiozoöid was transferred from sea water to the experimental solution and letter *B* an interval of 2 hours when the animal was not under observation. The length of the experimental time limited by these two letters in the graph equals approximately 40 minutes of continuous observation.

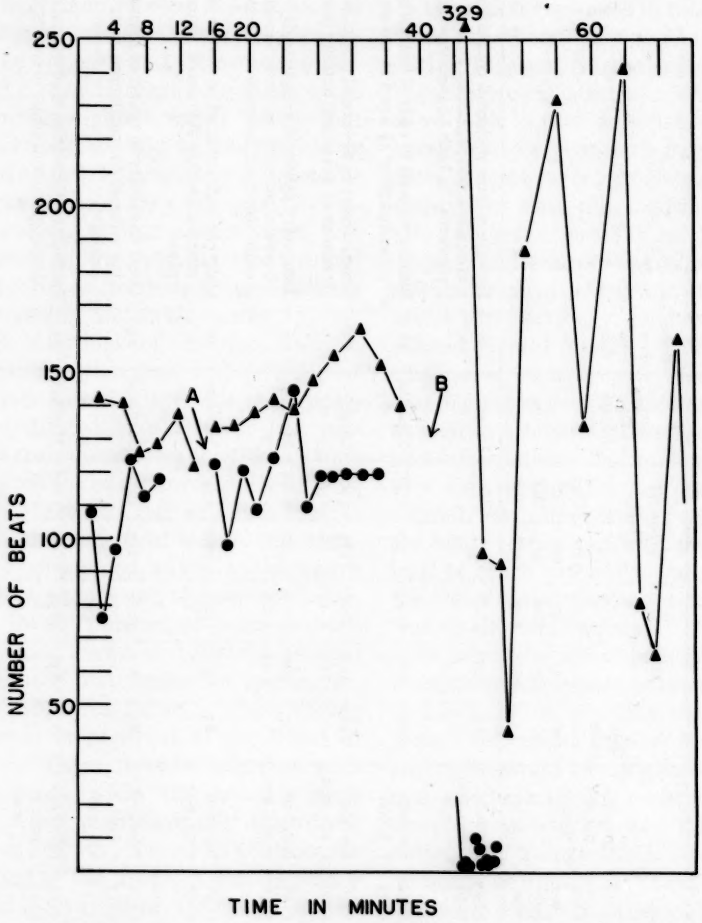

FIG. 1.—Graph showing the effect of nicotine 1:500,000 on the number of beats in both phases of the *Perophora* heart. Case B: *A*, point at which the animal was transferred from sea water to the experimental solution; *B*, an interval of 2 hours when the animal was not under observation. Triangles represent counts of the number of beats in consecutive abvisceral phases; circles represent counts of advisceral phases.

EXPERIMENTAL

Nicotine.—Very low concentrations (1:500,000, Case B, Fig. 1) at first increased the number of beats in both phases but subsequently reduced the

number in the advisceral to counts of 1–10 and provoked further high counts and an intensification of irregularity in the abvisceral phase. This type of reaction has been interpreted in previous experiments as pointing to an excitation of abvisceral dominance (Waterman, *loc. cit.*; Prosser, 1942). During the longer series of beats in the abvisceral phase, temporary cessation of heart activity was frequent, lasting from 5 to 35 seconds. Toward the end of the period of observation the length of time consumed during reversals increased to 10–25 seconds. In Case C (1:300,000) an effect was obtained which was similar to that in Case B, only the reaction occurred more quickly. Further evidence of excitation by lower concentrations, i.e., increase in the number of beats in either phase over the normal number, was indicated by the absence of depression, which follows transfer from one solution to another, as in the case of sea water alone.

Concentrations of 1:50,000 (Case A, Table 1) and 1:100,000 depressed the counts in both phases from the beginning of the experiment, the heart center responsible for the advisceral phase being most strongly and quickly affected. The heart became partly contracted and contained little blood (cf. pilocarpine below). The height of the pulsation waves was reduced. After 15–30 minutes' exposure at these concentrations, activity was reduced to pulsations at both ends of the tubular heart, which were irregular in origin and failed to carry the length of the heart. Animals in this condition responded to pricking, were generally extended, were slower in relaxing after tight contraction, and exhibited the greenish coloration of the blood which is peculiar to *in situ* coagulation in ascidians. Outwardly they appeared to recover after transfer to sea water, although it took several hours for the heart to approach the type of activity recorded for the fresh animal.

However, a minor nicotine effect may persist several days, possibly becoming permanent (cf. Davenport, 1941, crab heart). This was not particularly noticeable in the activity of the whole animal or its heart following return to sea water but appeared after repeated tests with nicotine on the same animal even 1 or more days apart. For example, lower concentrations of nicotine were effective, depression occurred more quickly, and there was seldom any increase in the number of beats in the abvisceral phase. These observations would appear to indicate that, within the experimental period at least, the effects of nicotine were not completely reversible.

Eserine.—Perophora was found to be sensitive to eserine. At a concentration of 1:5,000 (Table 1), activity was reduced to shallow unco-ordinated pulsations at each end of the heart after about 10 minutes' exposure; while in a 1:200,000 solution (Case B, Fig. 2) the number of abvisceral beats was slightly increased during the first 25 minutes of exposure, followed by an intensification of the typical irregularity in heart action which is peculiar to ascidians. The behavior during the advisceral phase corresponded to that during the abvisceral, except that the period of intensified irregularity included lower counts rather than higher ones. However, the amount of depression was conspicuously less than that for higher concentrations. Not all hearts reacted in this manner. As seen in Figure 3 (Case D) a concentration of 1:250,000 provoked slight increase in the abvisceral counts, slight depression of the advisceral number, and little, if any, intensification of irregularity between successive counts in both phases.

After strong physical stimulation or during transfer of the animal from one

solution to another, the heart may stop beating. Like acetylcholine, eserine stimulated a heart in this condition to resume activity more quickly and with less initial irregularity of action than was the case with sea water. The animals remained expanded during the experiments, and *in situ* clotting of the blood occurred only when high concentrations reduced heart action to unco-ordinated beats. This reaction is similar to that described above for nicotine and is characteristic of the reaction to high concentrations of all drugs tested which were toxic. Shortly before this stage was reached, the circulation stopped in the stolon as well as in the body. Normal activity returned slowly upon replacement of the eserine solution with fresh sea water, but further exposure caused death. "Even after all heart activity had ceased, the animal continued for several minutes to respond to pricking with a fine glass needle. Siphons and test exhibited slow contractions."

If an animal was first exposed to eserine (1:300,000) for 15–30 minutes or longer, lower concentrations of acetylcholine (1:100,000–1:1,000,000) were effective than in the case of the fresh animal. Furthermore, the acetylcholine effect, i.e., stimulation, was prolonged by many minutes. Thus eserine potentiates the action of acetylcholine on the ascidian heart.

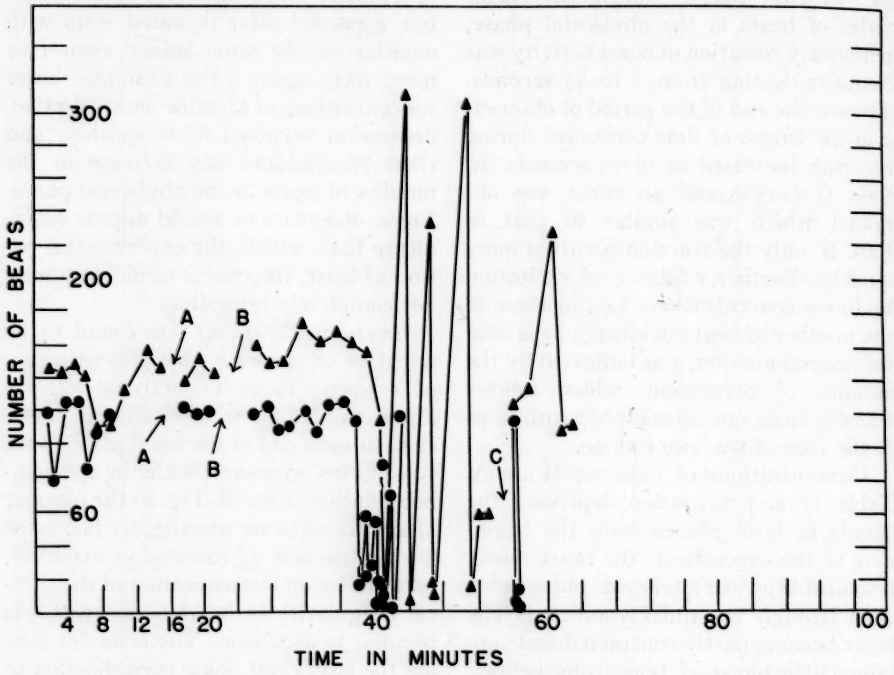

FIG. 2.—Graph showing the effect of eserine 1:200,000 on heart activity. Case B: *A*, animal transferred to fresh sea water; *B*, transferred to eserine solution; *C*, interval of 84 minutes of non-observation.

Pilocarpine.—Relatively large doses of pilocarpine had to be used before any effects were obtained. Of 16 animals studied, only 2 will be described (Table 1). A

solution of 1:1,000 (Case A, Fig. 4) caused immediate depression of the advisceral phase, while the increased length of the abvisceral phases attained two high peaks of 269 and 260 beats, respectively, within the first 16 minutes' exposure before the latter phase was depressed to the level of the advisceral. Toward the end of the experimental period, frequency of reversal increased, amplitude of contraction decreased (height of pulsation waves), and the amount of blood passing through the heart was greatly reduced. Subsequent activity became reduced to irregular pulsations at the ends of the heart, which were almost three times as numerous at the stolon end as at the visceral end. When the pulsations finally ceased, the heart was in a partly contracted state with few blood cells in the lumen. Later the heart of this particular specimen resumed beating in sea water. Figure 4 also illustrates the variation in the length of both phases of a normal animal as observed during a period of 48 minutes. With reference to the animal itself, the expanded state with extended siphons was maintained for the most part throughout the experiment with only occasional short periods of contraction lasting at most but several minutes each.

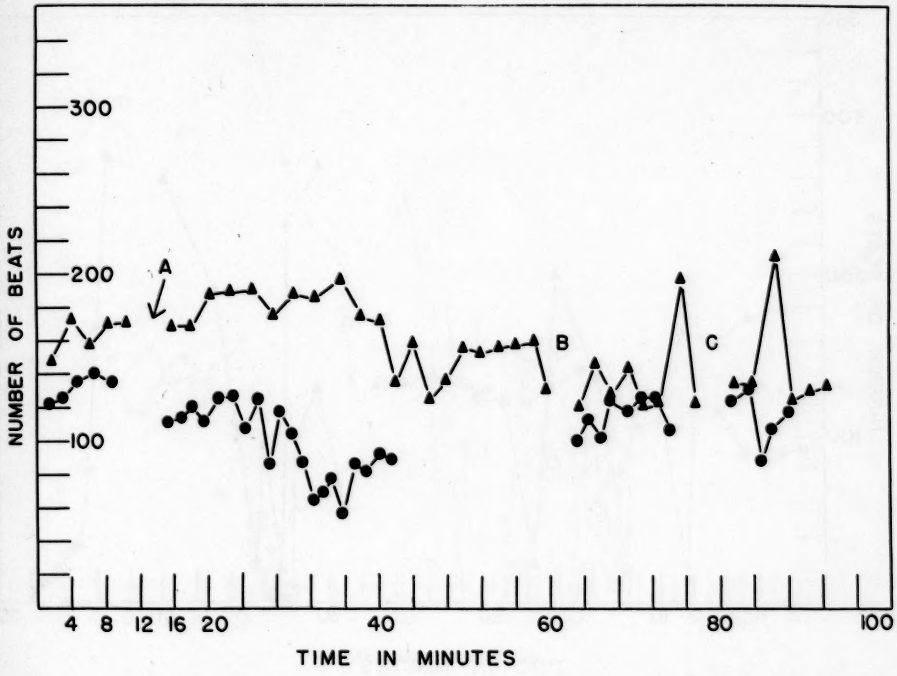

FIG. 3.—Graph showing the effect of eserine 1:250,000. Case D: *A*, transferred to eserine solution; *B* and *C* represent intervals of 4 and 5 hours, respectively, when the animal was not under observation.

Case H, Figure 5, illustrates the effect of a 1:5,000 solution of pilocarpine. Immediately after transfer to pilocarpine, the advisceral counts became slowly depressed to a low level of 22–40 beats, and this condition was maintained with but

slight variation during the succeeding 2 hours of interrupted observation. In contrast to this, the abvisceral counts immediately increased to a high peak of 356 beats, from which level the counts in succeeding phases gradually were reduced to a final level corresponding closely to that reached by the advisceral phase. Accompanying this depression in both phases, there was an increase in the variability of the counts for the abvisceral. Heart rate showed little variation and then chiefly at the time of reversal, when it was slightly slower, and the interval between reversals varied between 1 and 11 seconds in length. In fact, no drug thus far studied appeared to influence heart rate to any noticeable extent except at the time of reversal and in the case of high concentrations, which were strongly depressive and quickly toxic. Lower solutions than those illustrated above were progressively less effective in influencing heart activity.

Atropine followed by pilocarpine.—Atropine alone depressed the advisceral counts to a lower level than that characteristic of the normal animal in sea water and increased variability in heart action, while in the beginning of the experiments it slightly increased the number of abvisceral beats in some phases. Concentrations of 1:2,500 and 1:5,000 produced immediate irregularity of behavior and stopped all activity within approximately 20 minutes. Case D (Fig. 6) shows the result of exposure to three different media: sea water, 1:20,000 solution of at-

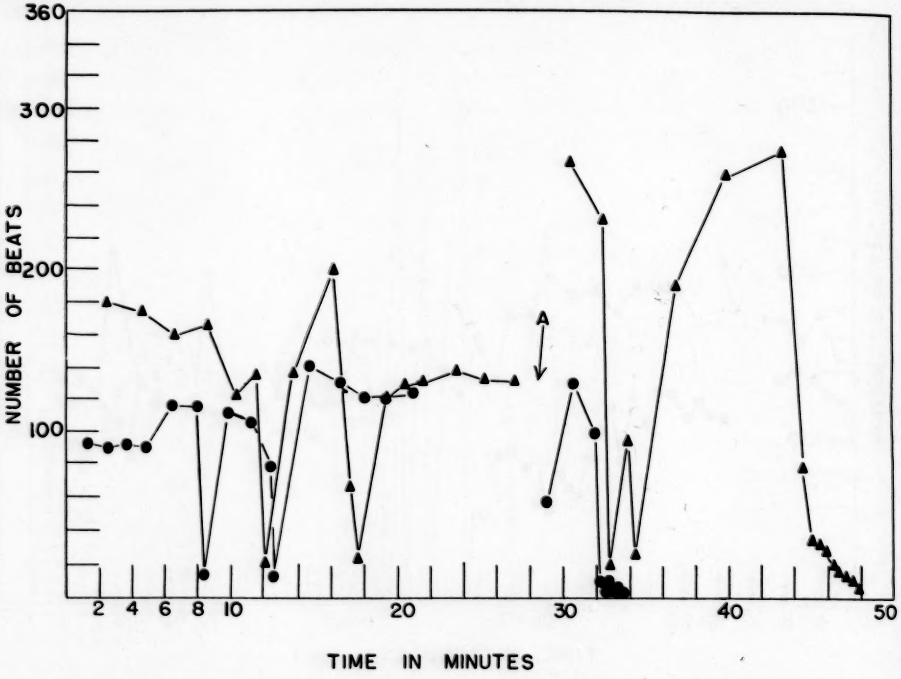

FIG. 4.—Graph showing the effect of pilocarpine 1:1,000 on the heart of *Perophora viridis*. Case A: *A*, point at which the animal was transferred from sea water to pilocarpine solution.

ropine in sea water, and 1:5,000 concentration of pilocarpine. Pilocarpine caused the following effects on heart action of the atropinized animal: further reduction of the advisceral counts to 1–5 beats, inhibition of any stimulatory effects upon abvisceral dominance, prolongation and intensification of variability in the abvisceral phase, and retardation of the depressive action upon the abvisceral phase. Even after many minutes' exposure to pilocarpine, counts in some of the abvisceral phases were as high as those recorded for the normal animal in sea water, although intervening counts were as low as 1–49 beats. One case only is reported here, but several repetitions upon animals of various sizes gave comparable results. Higher concentrations of atropine resulted in progressively greater depression of the abvisceral beats after the animal was transferred to the pilocarpine solution. It is concluded from the above that previous exposure to atropine inhibits the stimulatory action of pilocarpine on the heart of *Perophora*.

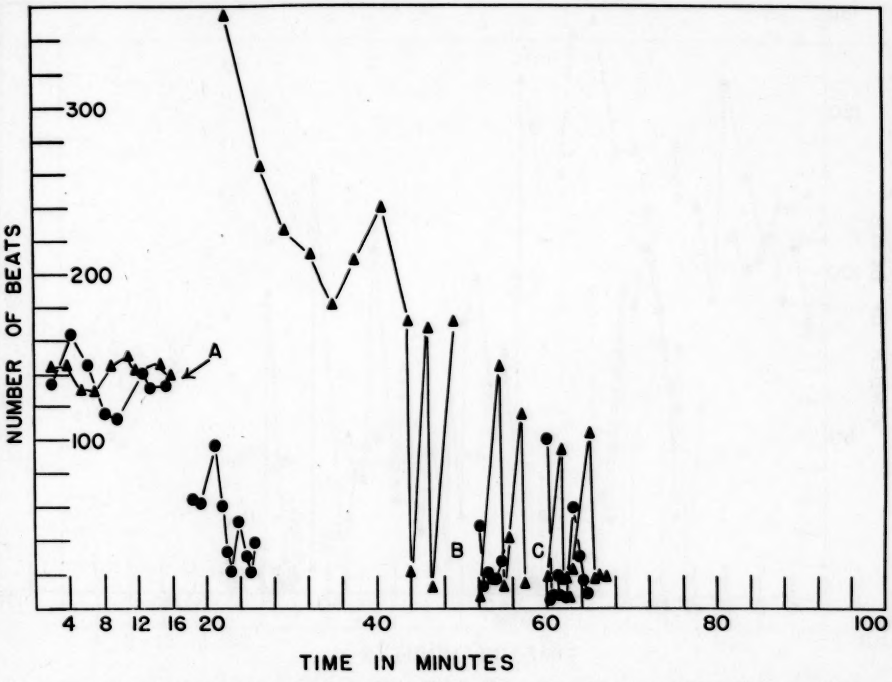

FIG. 5.—Graph showing the effect of pilocarpine 1:5,000. Case H: *A*, transferred from sea water to pilocarpine solution; *B* and *C*, intervals of 26 and 64 minutes, respectively, when the animal was not under observation.

The lessening antagonism between a given concentration of pilocarpine (1:5,000) and lower concentrations of atropine is illustrated by Figures 7 and 8. In Figure 7 (Case C) it can be seen that the depressive action of atropine on the advisceral phase was likewise intensified by subsequent exposure to pilocarpine. The number of beats was immediately reduced to a very low level. On the other hand, the record of the abvisceral counts showed the characteristic pilocarpine re-

action, and the number of beats ranged from 7 to 208. However, none of the higher counts exceeded those from the fresh animal. When a still lower concentration of atropine (1:50,000) preceded the pilocarpine solution (Fig. 8, Case K), even after several hours' exposure. Thus, the lower the atropine solution, the greater is the stimulating action of pilocarpine.

Atropine followed by acetylcholine.—If an atropinized animal was treated with acetylcholine of the same concentration

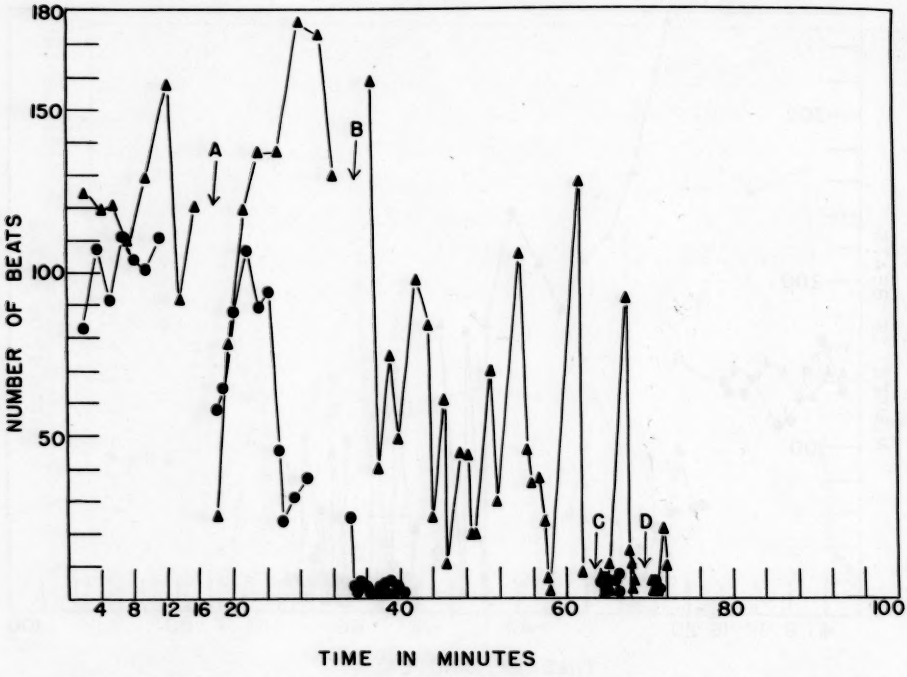

FIG. 6.—Graph showing the heart-beat record of an animal in sea water, in atropine 1:20,000, and in pilocarpine 1:5,000. Case D: *A*, point at which the animal was transferred from sea water to atropine; *B*, transferred to pilocarpine solution; *C* and *D*, intervals of 42 and 192 minutes when the animal was not observed.

counts in some of the abvisceral phases went as high as 283, which far exceeded any of those for the fresh animal. In this particular case there was an interval of approximately ½ hour between the time of transference to pilocarpine and the appearance of its effects. Further, some of the advisceral counts were higher than those shown in Figure 7. These results are comparable to those obtained with pilocarpine alone and were in evidence (but not toxic) as the atropine, there was, at first, no response characteristic of this drug. However, after 15–30 minutes the abvisceral phases showed occasional high counts, and irregularity of activity was slightly intensified. Fresh atropine eliminated these latter effects. Concentrations of atropine greater than 1:50,000 deceased the acetylcholine effects. Although briefly presented here, these results show that atropine temporarily

blocks the action of acetylcholine on the heart of *Perophora* (cf. Hamilton, 1939, for the grasshopper heart).

DISCUSSION

This paper presents another physiological study of drug action on the ascidian heart and attempts to furnish additional evidence for the conclusions derived from a previous study (Waterman, 1942) that (1) the heart of *P. viridis* has a nervous structure of its own, (2) a cholinergic type of chemical mediation is involved in heart action, and (3) the provoked increase in the length and variability of the abvisceral phases by acetylcholine, mecholyl, and adrenalin represents a stimulation of the effective heart center with its pacemaker mechanism. This increase is not brought about merely by a depression of the advisceral center, because the latter effect occurs independently while the abvisceral phases are passing through both stimulated and depressed periods. Mere transfer of an animal from one dish of sea water to another simply depresses both phases for a very brief time. With effective concentrations of adrenalin, the dominance of the abvisceral phase may be prolonged for hours, and the heart beats in this one direction only. Atropine, strychnine, and colchicine are depressive on both phases without discrimination.

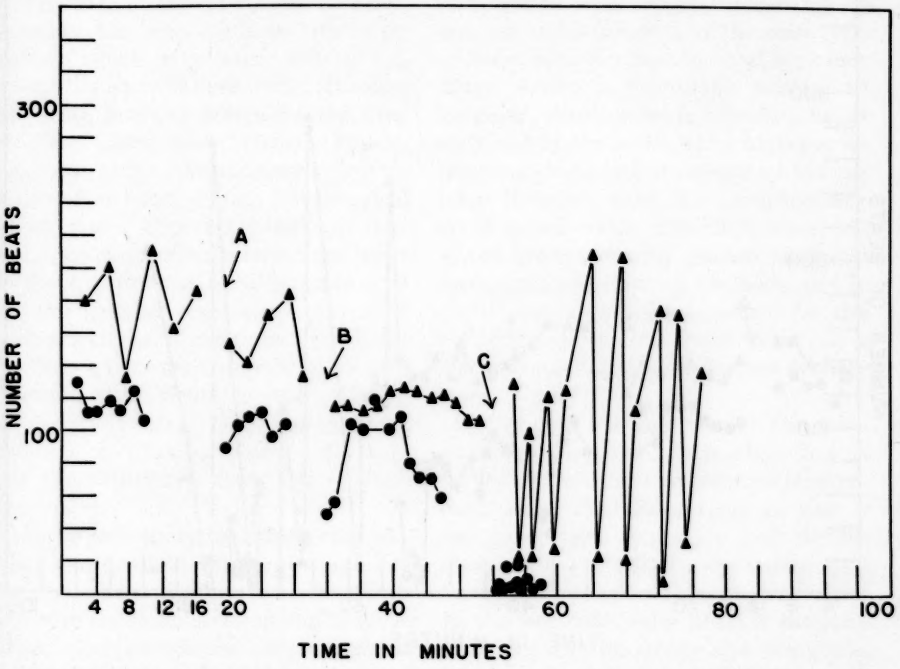

FIG. 7.—Effect on the heart-beat record of an animal exposed to sea water, to atropine 1: 30,000, and to pilocarpine 1: 5,000. Case C: *A*, transferred from sea water to fresh sea water; *B*, transferred to atropine; *C*, transferred to pilocarpine.

This previous study also showed that all the drugs tested have a depressive ac-

tion on (shorten) the length of the advisceral phase in proportion to the concentration employed, depress both heart centers in high concentrations, and in low concentrations are not injurious, providing exposure has not been too long or the heart has stopped beating. As the inas well. In this case the pulsations originate at the heart centers with apparently just as strong an action as before but fail to be carried across to the opposite end. Prosser and Zimmerman (1941) have suggested that those animal hearts which are accelerated by acetylcholine are neu-

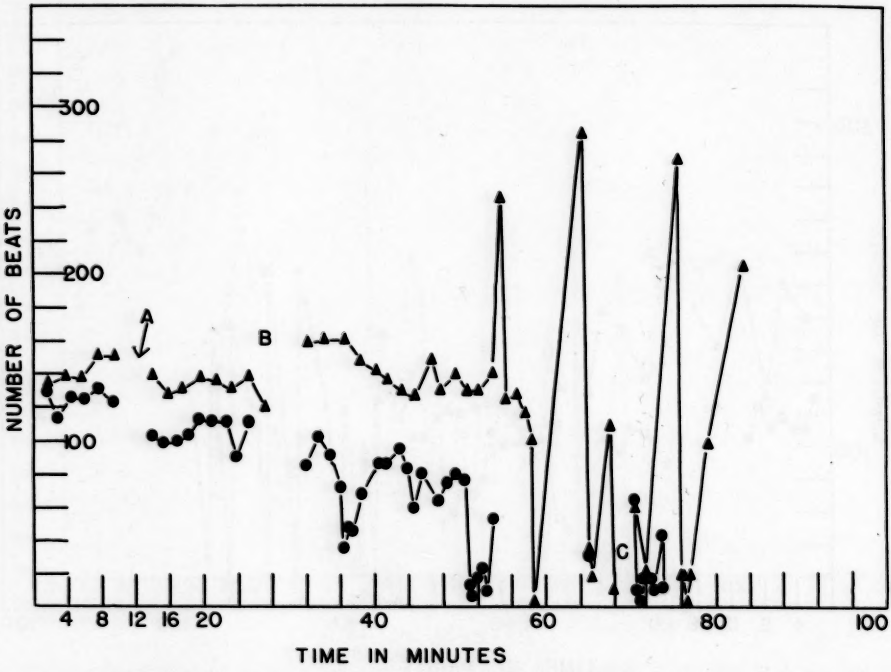

FIG. 8.—Record of the heart action of an animal in sea water, in atropine 1: 50,000, and in pilocarpine 1: 5,000. Case K: *A*, transferred to atropine solution; *B*, transferred to pilocarpine; *C*, represents an interval of approximately 3½ hours when the animal was not observed.

tersiphonal ganglion probably does not exert much, if any, controlling action on the heart because the isolated ascidian heart continues to beat and reverse, the primary effect of low concentrations of the drugs is directly upon the heart centers and their pacemaker mechanisms. It is only in high concentrations, which eventually reduce heart action to uncoordinated pulsations restricted to the ends of the heart, that the conductile and contractile mechanisms become impaired

rogenic (decapod crustaceans, insects, *Limulus*, *Arenicola cristata*, *Talorchestia*); while those which are inhibited or unaffected are myogenic (*Daphnia*, mollusks, adult vertebrates). The fact that the ascidian heart responds to low concentrations of acetylcholine and especially adrenalin leads to the inference that it is innervated (cf. Marrazzi, 1939).

However, any conclusions regarding structure to be derived from drug action must eventually be substantiated by

morphological studies. As seen in the living specimen, the heart of *Perophora* is a tubular structure, which tapers slightly at each end. It is marked by a transverse constriction in the center of the tube and by a spiral structure of the wall. The small size of *Perophora* has made histological study of its heart extremely difficult. However, some histological study has been made by others on ascidians which have larger hearts, and nervous tissues have been found (Hunter, 1902, 1903, heart of *Molgula manhattensis;* Ten Cate, 1928, *Ciona;* Fedele, 1923*a*, *b*, 1927; Alexandrowicz, 1913). On the other hand, certain physiological studies would appear to indicate that there is no connection between the heart and the intersiphonal ganglion or at least that the ganglion exerts no control of heart activity and even that the heart may lack a nervous structure of its own (Ransom, 1884; Schultze, 1901; Hecht, 1918, *Ascidia atra;* Day, 1919; Bacq, 1935*a*, *b*, *c*, *Ciona*). These references have been discussed elsewhere (Waterman, 1942).

The present study has shown that nicotine, eserine, and pilocarpine quickly penetrate the body tissues of *Perophora* even with the siphons closed and that the effects upon heart action are comparable to those described above for acetylcholine, mecholyl, and adrenalin. The heart cells are not injured by these drugs, as the effects are reversible in sea water. However, with nicotine, recovery is not complete immediately because repeated exposures tested on the same animal at daily intervals provoked a response which was somewhat different from that given by the fresh animal. For instance, lower concentrations are effective, depression occurs more quickly, and there is seldom any increase in the abvisceral counts. These drugs confirm the conclusion that the two pacemakers are differentially susceptible. Increase in the abvisceral counts may be initiated immediately after exposure to the test solution (nicotine, Fig. 1; pilocarpine, Figs. 4 and 5) or after a short lag period, which may be as long as 16 minutes in length at the concentrations tested (atropine, Fig. 7; eserine, Fig. 2). The period of stimulation seldom lasts longer than about 15 minutes and is followed by the same type of heart behavior as described for other drugs. Action is eventually reduced to irregular, unco-ordinated pulsations at each end of the heart when higher solutions are employed or exposure has been long. Recovery from this condition may occur in sea water. The little blood left in the heart is simply pushed back and forth, and elsewhere in the body and in the stolon circulation ceases. *In situ* clotting follows, the blood takes on a greenish color, and soon all heart activity stops.

In general the reaction of the *Perophora* heart to nicotine resembles that reported for certain other animals (Davenport, 1941; Hamilton, 1939) in that it can cause both excitation and depression, or even inhibition, depending upon the concentration and length of exposure. In the isolated *Cancer* heart it produces an increase in frequency and amplitude, but not in *Perophora*. Hunter (1903) found that nicotine depresses the pulsation periods in the heart of *M. manhattensis*, especially the abvisceral phase, and provokes irregularity. From studies in which the ganglion was removed he concluded that nicotine acts on the junction of the inhibitory fibers and the ganglion cells in the heart, provided that such an apparatus exists in this animal. Kruckenburg (cited by Hunter) found that nicotine reduces only the duration of the advisceral-pulsation period in the *Salpae* by acting on the hypobranchial center, which, he concluded, indicates

that reversal is reflex and is brought about through the ganglion. Schultze (1901) reported that high concentrations (1:10,000 and 1:25,000) caused immediate and strong reduction of the number of beats in both phases in *Salpa africana-maxima* and also great irregularity. A low concentration (1:100,000) caused slight increase in the length of the pulsation period, a slight depression in the abvisceral, and acceleration in the advisceral, direction.

Acetylcholine has an excitatory action on the heart of *Perophora* (Waterman, *loc. cit.*) and arthropods (Welsh, 1939*a*, *b;* Hamilton, 1939) but inhibits the hearts of mollusks (Prosser, *loc. cit.*) and vertebrates. Eserine prevents the enzymatic hydrolysis of acetylcholine by cholinesterase, and it sensitizes certain muscle tissues to acetylcholine. Welsh (1939*a*, *b*) found that eserine increased the frequency of the crustacean heart, but no evidence of this was found in the present study (cf. Davenport, 1941, for crayfish heart). In *Perophora* eserine produces effects on the heart which resemble in certain respects those described for acetylcholine, except that the period of intensified irregularity of the number of beats in both phases was preceded by a short period of consistently high counts, in which depression was delayed. Eserine seems, therefore, to be milder in its excitatory action than acetylcholine, but the effects last longer. Eserine potentiates the action of acetylcholine on the ascidian heart—a fact which, according to Brown (1937), is strong evidence of cholinergic transmission. This reaction, together with the acetylcholine-like action of the drug, would appear to confirm the existence of cholinesterase in ascidian tissues, as reported by Bacq, though he failed to find any trace of acetylcholine (Bacq, 1935*b*).

According to Bacq, the physiology of adult tunicates is as different from that of vertebrates as from that of invertebrates, while Skramlik (1930) believes that tunicates have a position both zoölogically and physiologically between mollusks and fishes. Prosser (1942, p. 157) states:

> According to the relation between acetylcholine acceleration and neurogenicity presented in this [Prosser's] paper, Waterman's evidence supports Hunter that at least some ascidian hearts are neurogenic. One must conclude either that the ascidians show aberrant mutation from the chordate line or else arose long before the main chordate characters were established. In support of the latter view is the fact that ascidian muscles contain the invertebrate arginine phosphate rather than the vertebrate phosphocreatine.

Bacq (1935*a*, *c*) found that acetylcholine has a strong effect and causes contraction of the muscles of the mantle, but the sensitivity of the muscle of *Ciona* was not increased by eserine (solution $1 \cdot 10^{-6}$). Bacq (1939) later found that eserine is somewhat toxic but does not increase the reaction of ascidian muscle to electrical stimulation, and that stimulation of eserinized muscle does not free a substance whose action is identical on the muscle of the leech to acetylcholine. He concluded that this points to the noncholinergic nature of nerve innervation of the longitudinal muscle of *Ciona intestinalis* and *Cynthia papillosa*.

Atropine antagonizes the action of acetylcholine in vertebrates and Crustacea (Welsh, 1939*a*, *b;* Hamilton, 1939; Davenport *et al.*, 1940; Davenport, 1941; etc.), presumably by competing for the receptor substances (Prosser, 1942), without preventing the response to nerve impulses. But in mollusks (Prosser, 1940, 1942, etc.) there are toxic effects, and no antagonism is found. Atropine likewise appears to block the excitatory action of acetylcholine on the heart of *Perophora*. Does this mean that the ac-

tion of acetylcholine in *Perophora* is muscarine-like? However, as indicated elsewhere in this paper, there is some resemblance between the initial action of both nicotine and acetylcholine. Nicotine temporarily excites the heart (it usually stimulates neurogenic hearts [Prosser, 1942]), but not to the same extent or for as long as acetylcholine, and eventually stops the heart in a semicontracted state. This evidence would seem to suggest that the two substances work at first in the same manner on the intact heart of *Perophora*. According to Prosser (1942), it is difficult, if not impossible, to classify acetylcholine effects on hearts as muscarine-like or nicotine-like. Pilocarpine likewise provokes initial excitation in terms of increased number of beats, etc., followed by depression over a long period of time, but relatively high concentrations are necessary to produce these reactions. Atropine antagonizes the action of pilocarpine on the heart of *Perophora* (cf. Bain, 1929, for crustacean hearts).

Various physical tests have failed to elicit responses similar to those described for these drugs. Loose or tight contraction of the normal animal in sea water neither changes the frequency of reversal, lengthens either phase appreciably, nor changes the rate of heart beat from that seen in the extended condition. Slight stimulation of the animal by touching the body or certain especially sensitive regions, like the siphons, provokes no change in heart action. Only occasionally does an animal change the direction of beating when shifted from one dish of sea water to another, and the resulting slight depression in both phases lasts for only a short time. Transfer from sea water to the experimental solution may sometimes cause a change in the direction of beating. When the animal is so strongly stimulated that circulation ceases, the heart stops beating, and *in situ* clotting occurs, then resumption of beating after transfer to another dish of sea water or a rest period may be in the opposite direction.

The evidence that (1) acetylcholine appears to have an excitatory action of a certain type, (2) eserine produces certain effects similar to those of acetylcholine and, furthermore, sensitizes the heart to lower concentrations of acetylcholine and prolongs the effects of the latter, and (3) atropine, which is said to prevent the muscarine-like action of acetylcholine, blocks the action of acetycholine and pilocarpine would seem to suggest that acetylcholine may play a part in the function of the *Perophora* heart and lends further support to the conclusion that the heart is neurogenic (Prosser, 1942). Since the evidence of drug action in the ascidian heart is still fragmentary and the problem of nervous structure in the heart still unsettled (Waterman, 1942), it may be premature to draw definite conclusions as yet concerning the effect of drug action on the nervous mechanism. However, the results of these studies are suggestive when a comparison is made with similar studies by others on many invertebrate hearts.

SUMMARY

1. The effects of eserine, nicotine, pilocarpine, pilocarpine preceded by atropine, and acetylcholine preceded by eserine or atropine on the intact heart of the compound ascidian, *P. viridis*, have been studied by exposing the whole animal to solutions of each drug in several concentrations to determine if a neurohumoral system (possibly cholinergic) of heart control is present.

2. Certain concentrations of nicotine, eserine, and pilocarpine immediately depress the number of beats in the advisceral phase but have an excitatory ac-

tion on the abvisceral phase by increasing the number of beats, intensifying irregularity, and increasing the frequency of reversal. These effects on the pacemaker mechanisms resemble those described previously for acetylcholine, mecholyl, and adrenalin. High concentrations or long exposures reduce the height of the pulsation waves, depress the number of beats in both phases, lengthen the time at reversal, slow the rate, depress the extent of relaxation, increase irregularity of contraction, and finally reduce activity to unco-ordinated pulsations at each end of the heart which fail to carry to the opposite end. These are effects primarily upon the conductile or contractile mechanisms or both.

3. The drugs penetrate the animal tissues rapidly, as shown by the speed of heart reaction, even with siphons closed. All produce only depressive effects on the advisceral phase. Results support a previous conclusion that the heart centers, which are responsible for the origin of the pulsations in the directional beating of the heart, are differentially susceptible to drugs.

4. Eserine excites a blocked heart to resume activity more quickly. In an eserinized animal lower concentrations of acetylcholine are effective, and the action is prolonged. Eserine excites the heart and in this respect is comparable to the action of acetylcholine, except that the former has a milder action and the excitation period thus produced is of longer duration.

5. Atropine temporarily blocks the action of both acetylcholine and pilocarpine. Does this mean that acetylcholine has a muscarine-like action? Some of the effects caused by nicotine resemble those of acetylcholine, which would seem to indicate that these two substances work at first somewhat in the same manner on the heart.

6. Evidence is discussed in support of the conclusion that acetylcholine may play a part in the normal activity of the *Perophora* heart and hence that a cholinergic system of heart control may be present. However, it is probably premature to draw definite conclusions as yet between drug action and nervous mechanism in ascidians.

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THE NITROGEN COMPOUNDS NECESSARY FOR GROWTH IN COLPIDIUM STRIATUM STOKES, WITH SPECIAL REFERENCE TO THE AMINO ACIDS[1]

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OSBORNE and Mendel (1914) stated: "The current trend of the investigation of the chemistry of nutrition is emphasizing the significance of the amino acids as the fundamental factors in problems in which hitherto the role of the proteins has been involved. The question of protein synthesis has now become a problem of the biochemical deportment of the amino acids." From this time onward the significance of the various amino acids in the preservation of the normal physiological processes of the organism was recognized as a subject for research.

According to Rose (1938), two methods of investigation were used almost exclusively in these researches. The first involved the use of purified incomplete proteins; the second, diets from which certain amino acids had been extracted by chemical methods.

While these methods were fruitful, the results obtained seldom solved any problem conclusively. As Rose (1938) points out, this lack of finality is the result of defects inherent in the methods. The results were difficult of interpretation, be-

[1] Gratitude is expressed to Professor S. O. Mast for suggesting the problem and for his advice and criticism during the course of the work. Appreciation is also expressed to Dr. Donald M. Pace for his helpful suggestions. Grateful appreciation is also extended to Merck and Company, E. R. Squibb and Sons, and the Distillation Products Company for the vitamins used in this study.

cause there were no very accurate means of detecting and quantitatively separating the various amino acids. Moreover, so little was known concerning the vitamins that it was difficult to maintain health and appetite in the experimental animals, and so they often died because of an avitaminosis rather than because of the lack of a necessary amino acid.

In 1930 Rose (1938) began a series of investigations to ascertain whether amino acids suffice for the nutrition of rats and, if so, which of them are necessary. By 1937 he found that amino acids suffice if the proper vitamins are present and that the following acids are necessary: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine.

The definite character of the results obtained by him is due to the care with which the amino acids were purified before the diets were prepared, for, as Rose and St. Julian (1932) had previously said, "Final judgment must await the results of feeding experiments involving the use of highly purified amino acids known to be entirely devoid of the compounds in question."

In all the experiments of Rose and his predecessors mammals were used. Schmidt (1938) says: "It must be emphasized that most of the data available as to the amino acid requirements of the animal organism have been secured with relatively few species and in particular with the rat as the experimental animal."

Pringsheim (1921) maintained that the flagellate *Chilomonas paramecium* will grow in a sterile culture medium containing glycine and an acetate but that it will not grow if the compounds containing nitrogen and carbon are less complex than these. Mast and Pace (1933), however, conclude that it can obtain these two elements from ammonia and carbon dioxide.

Lwoff (1924) stated that *Glaucoma piriformis*, a ciliate, requires peptides or even more complex protein derivatives for growth. Elliott (1935) concludes that single amino acids do not satisfactorily support the growth of *Colpidium striatum* or *C. campylum*. Hall and Elliott (1938) maintain that incomplete proteins do not support growth in *C. striatum* and that the addition of the missing amino acids does not suffice unless yeast extract, liver infusion, or tryptone is also added.

The results in hand therefore indicate that some flagellates can obtain nitrogen from ammonia and that some mammals can obtain it from amino acids, but that ciliates require more complex compounds. Rose found, as stated above, that amino acids suffice for growth in rats only if various vitamins are present. It may well be, then, that the failure to obtain growth of ciliates on amino acids has been due to lack of vitamins. This matter is considered in the following experiments.

MATERIALS

The colpidia used in this study were raised from a stock obtained from William J. Bowen which had been cultured for some time in a sterile medium consisting of 1.5 per cent tryptone and 0.2 per cent monobasic potassium phosphate in water. Stock cultures were maintained on sterile agar slants consisting of 2 per cent proteose peptone in 1.5 per cent agar in water.

The colpidia used were obtained from these stock cultures as follows: The stopcock in the side arm of a special glass vessel (Fig. 1) was closed, and the main compartment nearly filled with an aqueous solution of anhydrous dextrose (M/10). It was then sterilized by autoclaving (at 20 pounds for 20 minutes). Many colpidia were removed from an

agar slant with a solution of 1.5 per cent tryptone by means of a long, sterile pipette and put into the side arm of the glass vessel. A short piece of sterile rubber tubing, plugged with cotton, was slipped over the open end of the side arm; the stopcock in the side arm was opened; and the culture fluid in it was forced by pressure into the bottom of the main compartment underneath the dextrose solution. The glass vessel was then put into a bucket of ice water. This caused the colpidia to come to the surface, from which they were transferred to sterilized test tubes. Since the specific gravity of the solution containing the dissolved proteose peptone and tryptone was greater than that of the dextrose solution, this manipulation resulted in the elimination of the proteose peptone and tryptone in the solution in which the colpidia were when they were put into the side arm. All the colpidia used in the following experiments were taken from dextrose solution in test tubes prepared in this way.

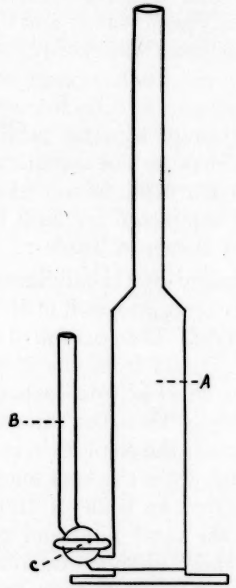

FIG. 1.—A special glass vessel used to eliminate amino acids, polypeptides, and other protein derivatives from the medium in which the colpidia were cultured. *A*, main compartment; *B*, side arm; *C*, stopcock.

The amino acids used in this study were the purest commercially obtainable. Some were produced by hydrolysis; others, by synthesis. The manufacturers gave assurance that each of the latter was free from other amino acids, but could not do the same for the former.

The amino acids prepared by hydrolysis, *l*-cystine, glycine, *l*-histidine, *l*-hydroxyproline, *l*-proline, *l*-tryptophane, and *l*-tyrosine were examined for the presence of other amino acids by some thirty qualitative tests. The results obtained indicated that these acids, although derived from natural sources, contained no other amino acids. This must not, however, be taken as an assertion that other amino acids were not present, but merely as a statement of their probable absence, for the tests were examined only roughly for their accuracy and sensitivity. There were no tests available for ascertaining the purity of isoleucine, lysine, methionine, or valine.

The inorganic chemicals used were Schering-Kahlbaum of the highest purity.

METHODS

EXPT. 1: GROWTH OF *C. striatum* IN A MIXTURE OF AMINO ACIDS

Elliott (1935) found that colpidia grow and reproduce well in a sterile solution of 1.5 per cent Bacto-tryptone. Tryptone is a partially hydrolyzed tryptic digest of casein. In attempting to grow colpidia in a mixture of amino acids, the best attack on the problem consequently seemed to be in the use of the amino acids present in casein (Table 1).[2]

[2] Beta-hydroxyglutamic acid, citrulline, and some other amino acids whose presence in casein is in doubt were not used in this study.

TABLE 1*

COMPOSITION OF THE CULTURE MEDIUM USED IN EXPERIMENT 1

Substances	Solubility (Gm/100 Ml.)	Per Cent in Casein	Gm/100 Ml. of Medium
Amino acids:			
dl-alanine	14.7	1.9	0.03499
d-arginine monohydrochloride	v.s.w.	3.8	0.06960
dl-aspartic acid	0.82	4.1	0.07550
l-cystine	0.011	0.3	0.00542
dl-glutamic acid monohydrate	v.s.w.	21.5	0.20072
Glycine	21.65	0.5	0.00915
l-histidine monohydrochloride	v.s.w.	2.5	0.04504
l-hydroxyproline	24.90	0.2	0.00370
dl-isoleucine	2.18	9.7	0.08420
dl-leucine	0.99		0.08420
dl-lysine monohydrochloride	v.s.w.	6.0	0.11049
dl-methionine	3.28	3.4	0.06261
dl-phenylalanine	1.42	3.9	0.07182
l-proline	11.51	9.0	0.16574
dl-serine	5.02	0.5	0.00920
dl-threonine	v.s.w.	.....	0.05000
l-tryptophane	1.14	2.2	0.04050
l-tyrosine	0.048	6.6	0.04000
dl-valine	7.44	7.9	0.14548
Vitamins:			
Vitamin-A ester concentrate			0.00002
Thiamin hydrochloride			0.00002
Riboflavin			0.00002
Nicotinic acid			0.00002
Calcium pantothenate			0.00002
Pyridoxine			0.00002
Ascorbic acid			0.00002
Viosterol in oil			0.00002
Carbohydrate:			
Anhydrous dextrose			0.01800
Inorganic salts:			
Calcium chloride			0.00555
Magnesium sulphate			0.00600
Dibasic potassium phosphate			0.00870
Dibasic sodium phosphate			0.01790

* The solubilities of the amino acids were calculated from Schmidt (1938). The quantities of the amino acids in the medium were calculated as follows: Cystine is the least soluble amino acid in casein. In order to obtain the strongest solution of these acids possible and at the same time retain the relative amounts of each as found in casein, the solubility of *l*-cystine was taken as a basis, and the rest of the amino acids were added to the medium in the same proportion as they occur in casein, except *l*-tyrosine and *dl*-glutamic acid monohydrate—because of the low solubility of the former and the expense of the latter, if pure.

Since, however, only 1 per cent of the casein hydrolysate consists of isoleucine and leucine together, the calculated quantity based on this amount was equally divided between them. The percentage of threonine in casein was not available; so an amount thought to be sufficient was added.

The percentages of the vitamins, inorganic salts, and carbohydrate in a tryptic digest of commercial casein were not available; so, again, quantities were added which were thought to be adequate.

Vitamin-A ester concentrate and viosterol in oil are difficultly soluble in water, but autoclaving caused them to form dispersions.

In preparing the medium used in this experiment and the other media based upon it, the amino acids were used in the same proportion in which they are present in casein. These media were prepared as follows: The concentrations were calculated, and the weighed amounts put into warm water and left until they had dissolved, after which vitamins, inorganic salts, and dextrose were added from previously prepared stock solutions. Each mixture was then diluted, and the hydrogen-ion concentration adjusted by the addition of sodium hydroxide. The osmotic concentration of each medium was also measured. The completed medium had the composition shown in Table 1.

One and eight-tenths milliliters of this solution were put into each of three vials and autoclaved. Then 0.2 ml. of a sterile culture of colpidia from one of the prepared tubes was put into each vial and left for 3 days. Then the three cultures were examined; the colpidia in each were counted; and, from the best one, 0.2 ml. was transferred to each of three vials containing the same kind and quantity of culture fluid as the preceding vials. This was repeated until there were nine successive cultures in all. The entire process was then repeated twice with amino acids and three times with an aqueous solution of 1.5 per cent tryptone and 0.2 per cent monobasic potassium phosphate. The results are presented in Table 2.

This table shows that in each of the six tests made the colpidia reproduced at a fairly uniform rate throughout the entire time the tests continued and that at the close of the experiment, 27 days after

it began, they still appeared to be growing and reproducing normally in all, but that, on the whole, the rate of reproduction was higher in the tryptone than in the amino acid culture fluid, the total average[3] per individual introduced per 3-day period being 12.8 for the former and 9.0 for the latter. The results presented therefore indicate that colpidia can grow and reproduce indefinitely in a solution which contains, other than vitamins, no nitrogen compounds more complex than amino acids but that they grow and reproduce more rapidly in a solution which contains more complex nitrogen compounds.

TABLE 2

GROWTH OF *C. striatum* ON AMINO ACIDS

The colpidia introduced into the first culture were obtained from a stock dextrose solution; those into the second, from the best of the first cultures at the end of the first period; etc. Temperature, 26° ± 2° C.; acidity, pH 6.16 ± 0.16; osmotic pressure, 2.65 ± 0.35 atmospheres.

Culture Fluid	Colpidia Introduced	Increase during Successive 3-Day Periods per Individual Introduced									Total Average
		First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	
Nineteen amino acids	59,000	0.5	1.5	7.6	10.9	12.3	10.9	7.7	8.4	11.2	.....
	2,000	0.9	8.3	11.3	5.9	14.0	12.1	12.4	8.6	9.5	.....
	400	5.3	9.0	8.4	10.6	7.0	8.3	22.0	8.6	10.4	9.0
Tryptone	59,000	11.6	9.1	12.0	22.5	9.1	8.8	16.8	11.5	10.2	.....
	2,000	7.0	7.0	8.6	16.3	17.7	15.8	14.7	19.0	12.5	.....
	400	6.3	7.1	10.7	18.1	13.7	19.3	14.7	13.2	9.9	12.8

Method used to keep the cultures sterile.—The cultures in Experiment 1 and all the others in these experiments were sterile. In order to obtain and preserve this sterility, the usual bacteriological precautions were observed in handling the cultures. Autoclaving at 20 pounds for 20 minutes was the means of sterilization. Pasteurization was tried, but it had no advantage over autoclaving and was not so convenient.

To give additional assurance of sterility, a few drops from each culture used in the experiments were put on nutrient agar slants and incubated for 2 weeks. As soon as it became apparent that a culture was infected with bacteria or contaminated with molds, it and all the cultures transferred from it were discarded. At first the proportion of cultures that became infected or contaminated was considerable; but, as the investigation progressed, fewer and fewer became infected, until at the close the number was negligible.

Method of counting.—The culture was shaken for 15 seconds, and a sample withdrawn with a capillary pipette graduated in 0.005 ml. Three portions were put separately on a slide, fixed, and stained with a 1:20 mixture of Sudan III and Bouin's fluid. The colpidia in each portion were then counted, and the total average for the three vials in each group calculated. The rate of increase per individual introduced[4] was computed from

[3] These total averages are not to be taken too seriously, since they are not statistically valid. They were calculated by adding together all the reproductive rates of a group and then dividing by the number of rates. They are included merely to furnish a very rough, but convenient, means of comparison.

[4] Whether growth occurred or not was the chief point of these studies. The rate of reproduction was used because it seemed to give a more clear-cut an-

these total averages. This rate is given to the nearest tenth in Table 2 and the following tables.

EXPT. 2: GROWTH OF *C. striatum* IN A MIXTURE OF AMINO ACIDS NECESSARY FOR THE GROWTH OF RATS

The culture medium used in this experiment was the same as that used in Experiment 1, except that alanine, aspartic acid, cystine, glutamic acid monohydrate, glycine, hydroxyproline, proline, serine, and tyrosine were omitted and the amount of dextrose added was increased to 1 gm. in order to keep the osmotic concentration of the medium approximately the same as that of Experiment 1.

TABLE 3

GROWTH OF *C. striatum* IN A MIXTURE OF TEN AMINO ACIDS

The ten amino acids used in this experiment are those which Rose (1938) found necessary for rats. Temperature, 26° ± 2° C.; acidity, pH 6.16 ± 0.16; osmotic concentration, 2.66 ± 0.35 atmospheres.

Culture Fluid	Colpidia Introduced	Increase during Successive 3-Day Periods per Individual Introduced			Total Average
		First	Second	Third	
Ten amino acids....	2,000	2.5	0.7	dead	.....
	1,500	1.7	0.9	dead	.....
	600	3.1	1.1	dead	.....
Tryptone..	2,000	6.9	10.5	17.1	.....
	1,500	3.9	11.9	14.1	.....
	600	5.6	16.7	12.9	11.1

The colpidia were cultured and counted as described above. The results obtained are presented in Table 3, which shows that for each of the three tests in the medium containing ten amino acids necessary for the growth of rats (i.e., arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine) the colpidia died out in a little over 6 days but that the rate of reproduction was higher during the first 3 days than in the medium containing nineteen amino acids (Table 2) but not so high as in the tryptone medium. These results indicate, therefore, that colpidia require more amino acids than do rats and that they obtain their nitrogen from certain specific amino acids rather than from amino nitrogen in general.

EXPT. 3: WHICH AMINO ACIDS ARE NECESSARY FOR GROWTH AND REPRODUCTION IN *C. striatum*?

Nineteen media were used in this experiment. All were the same as those used in Experiment 1, except that one of the amino acids was omitted in each medium and that the amino acid omitted was not the same in any two of the media. Each of the nineteen media prepared therefore lacked a different amino acid.

Colpidia were cultured in these media and in tryptone and counted as in Experiment 1, except that the period between transfers was 5 days and the experiment was continued for 50 days. The results obtained are presented in Table 4. This table shows that the colpidia did not survive more than 5 days

swer to this question than did the density of population. It was calculated by dividing the number of colpidia introduced into the number of colpidia present in the culture at the end of a given period. It follows, therefore, that the number of colpidia present at the end of a given period is the product of the reproductive rate and the number of colpidia introduced. Since all cultures had a volume of 2 ml., the density of population is obtained by dividing the number of colpidia present at the end of a given period by 2. For example, in Table 2 there were 59,000 colpidia introduced into the first amino acid culture, and the reproductive rate was 0.5. Hence, the density of population at the end of the first period was 14,750 colpidia per milliliter. The quantity 0.2 ml. of the first culture was introduced into the second; this is equivalent to 2,950 colpidia. From this figure and the reproductive rate the density of population can be calculated, and so forth.

TABLE 4

AMINO ACIDS NECESSARY FOR GROWTH OF *C. striatum*

Temperature, 26° ± 2° C.; acidity, pH 6.16 ± 0.16; osmotic concentration, 2.66 ± 0.35 atmospheres.

Culture Fluid	Colpidia Introduced	Increase during Successive 5-Day Periods per Individual Introduced										Total Average
		First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	Tenth	
Nineteen amino acids minus *dl*-alanine	2,200	0.9	dead									
	600	5.0	dead									
	600	3.5	dead									
Same minus *d*-arginine monohydrochloride	2,200	dead										
	600	3.0	dead									
	600	3.5	3.8	dead								
Same minus *dl*-aspartic acid	2,200	0.4	dead									
	600	1.6	dead									
	600	3.0	2.2	10.0	28.0	dead						
Same minus *l*-cystine	2,200	dead										
	600	dead										
	600	dead										
Same minus *dl*-glutamic acid monohydrate	2,200	1.1	dead									
	600	2.5	dead									
	600	3.5	3.5	dead								
Same minus glycine	2,200	0.8	dead									
	600	4.2	dead									
	600	3.5	1.4	dead								
Same minus *l*-histidine monohydrochloride	2,200	0.6	7.7	25.0	17.4	12.9	6.5	2.6	66.6	11.3	6.7	
	600	19.5	6.5	9.6	13.0	9.5	11.0	8.0	10.9	9.1	11.4	
	600	3.7	10.5	14.4	6.4	19.0	11.5	10.7	7.4	14.2	13.5	12.6
Same minus *l*-hydroxyproline	2,200	1.4	dead									
	600	3.3	dead									
	600	1.7	2.0	dead								
Same minus *dl*-isoleucine	2,200	1.2	dead									
	600	dead										
	600	1.8	dead									
Same minus *dl*-leucine	2,200	0.3	15.7	18.6	12.0	6.1	11.8	2.3	4.0	12.5	11.3	
	1,500	3.0	10.0	8.2	10.0	8.4	9.6	9.6	10.0	10.7	11.3	
	1,500	18.7	8.2	9.1	10.4	9.1	12.5	10.0	8.4	10.9	10.4	13.1
Same minus *dl*-lysine monohydrochloride	2,200	8.0	5.4	9.3	10.3	2.1	20.0	23.5	10.3	9.4	10.4	
	600	2.8	10.0	11.8	12.5	24.0	12.5	11.9	8.1	8.9	12.5	
	600	3.3	16.5	8.3	7.8	12.8	14.8	12.7	9.4	12.3	8.1	10.3
Same minus *dl*-methionine	2,200	0.2	dead									
	600	1.6	dead									
	600	0.8	8.0	dead								
Same minus *dl*-phenylalanine	2,200	1.1	2.5	dead								
	400	2.5	20.0	dead								
	400	3.7	4.7	5.7	10.0	12.5	12.0	33.3	10.5	dead		

TABLE 4—*Continued*

Culture Fluid	Colpidia Introduced	Increase during Successive 5-Day Periods per Individual Introduced										Total Average
		First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	Tenth	
Same minus *l*-proline	2,200	1.4	2.3	dead								
	400	dead										
	400	4.5	dead									
Same minus *dl*-serine	2,200	0.2	dead									
	600	1.6	dead									
	600	1.5	dead									
Same minus *dl*-threonine	2,200	0.9	8.0	7.5	11.6	2.8	22.5	5.5	20.0	19.0	4.7	
	600	1.7	4.8	14.0	10.7	9.3	12.1	8.8	12.6	11.0	11.9	
	1,500	0.9	14.3	11.5	9.1	19.0	11.5	10.6	7.4	14.2	7.8	9.2
Same minus *l*-tryptophane	2,200	0.7	10.0	dead								
	400	5.7	7.3	15.8	dead							
	1,500	1.3	6.0	21.6	dead							
Same minus *l*-tyrosine	2,200	0.2	dead									
	600	1.3	17.5	4.3	16.6	21.0	1.9	dead				
	400	6.0	1.2	dead								
Same minus *dl*-valine	2,200	1.3	3.3	dead								
	400	6.2	3.6	dead								
	400	6.0	1.3	26.6	dead							
Tryptone	2,200	2.5	23.4	15.3	12.0	9.1	9.7	15.0	13.8	15.9	24.2	
	1,500	3.2	17.0	19.7	21.4	14.9	13.7	9.8	11.4	10.6	21.1	
	400	7.7	11.2	18.6	25.1	7.0	18.8	19.4	33.3	11.2	23.0	15.3

with cystine lacking; 10 days with alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, isoleucine, methionine, proline, or serine lacking; 15 days with phenylalanine, tryptophane, tyrosine, or valine lacking; but that with histidine, leucine, lysine, or threonine lacking they were still vigorous at the close of the experiment, i.e., after 50 days.

Comparison of the results presented in Table 4 with those in Table 2 shows that the growth of colpidia in the media lacking histidine, leucine, and lysine was slightly better than that observed in the medium containing nineteen amino acids but not quite so good as that in tryptone. Growth in the medium lacking threonine was probably not different from that in the former medium.

After the colpidia had died out in the different media, the amino acid lacking in each and more colpidia were added. They grew as well in these supplemented media as they did in the first medium containing nineteen amino acids.

These results, therefore, indicate that colpidia require for growth and reproduction, together with vitamins, etc., the following amino acids: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, hydroxyproline, isoleucine, methionine, phenylalanine, proline, serine, tryptophane, tyrosine, and valine; they do not require histidine, leucine, lysine, or threonine.

EXPT. 4: THE AMINO ACIDS NECESSARY FOR GROWTH IN *C. striatum*

The results of Experiment 3 indicate, as stated, that fifteen out of nineteen amino acids present in casein are neces-

sary for growth in colpidia, but do not show that these are the only amino acids necessary. The following experiment concerns this.

A culture medium was prepared, as described above, containing the fifteen amino acids listed as necessary in the preceding experiment. Colpidia were cultured in this medium as previously described. The results are presented in Table 5, which shows that in the medium containing the fifteen amino acids found to be necessary for growth in colpidia there was no indication of reduction in the rate of reproduction during the 50 days that the experiment continued, but that the rate was considerably lower than it was in the culture medium containing tryptone, being 9.4 per individual per 5-day period in the former and 12.9 in the latter. These results indicate, therefore, that no amino acids except the fifteen used are necessary for growth in colpidia and that they can supply the kind of amino nitrogen required by this organism.

TABLE 5

GROWTH OF *C. striatum* IN A MEDIUM CONTAINING FIFTEEN AMINO ACIDS

All fifteen amino acids are necessary for indefinite growth (see Table 4). Temperature 26° ± 2° C.; acidity, pH 6.16 ± 0.16; osmotic concentration, 2.66 ± 0.35 atmospheres.

Culture Fluid	Colpidia Introduced	Increase during Successive 5-Day Periods per Individual Introduced										Total Average
		First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	Tenth	
Fifteen amino acids	2,000	4.3	6.6	7.6	13.1	10.8	14.8	9.2	8.8	10.3	11.6	.....
	1,500	4.6	7.4	2.5	12.5	7.1	9.2	13.6	8.0	13.6	6.3	.....
	600	8.8	9.0	1.2	8.3	12.0	13.3	13.7	9.1	8.0	15.0	9.4
Tryptone	2,000	7.4	11.3	9.8	9.8	13.1	10.9	15.7	21.9	18.5	10.1	.....
	1,500	4.0	9.5	11.1	12.9	12.0	13.7	19.8	13.7	10.9	17.3	.....
	600	7.9	10.1	14.1	15.6	13.9	9.0	14.8	20.1	19.7	15.5	12.9

EXPT. 5: GROWTH OF *C. striatum* IN MEDIA CONTAINING ONLY ONE AMINO ACID

Elliott (1935) investigated the growth of colpidia in different cultures each containing one amino acid. He did not, however, use all the amino acids present in casein, nor did he add vitamins. He maintains that none of the amino acids which he used supports growth in colpidia.

In the following experiment nineteen media were prepared, each containing only one of the nineteen amino acids found in casein but all the vitamins and other substances used in the media in the preceding experiments. The amount of dextrose in these media was increased to 1.8 gm. in order to keep the osmotic concentration at approximately 2.66 atmospheres.

The colpidia were cultured and counted, as described above, until they died out. Colpidia were also cultured concurrently in a medium containing dextrose (M/10), vitamins, and inorganic salts but no amino acids and in another medium containing tryptone. The results are presented in Table 6. These results show that the colpidia lived and reproduced for 10 days in the media containing aspartic acid, glutamic acid, isoleucine, leucine, methionine, phenylalanine, tryptophane, and tyrosine, respectively; 15–20 days in those containing lysine, proline, valine, alanine, cystine,

TABLE 6

GROWTH OF *C. striatum* IN MEDIA CONTAINING BUT ONE AMINO ACID

The concentration of the amino acid present was M/100 unless otherwise stated. Temperature, 26° ± 2° C.; acidity, pH 6.16 ± 0.16; osmotic concentration, 2.66 ± 0.35 atmospheres.

Amino Acid Present in Medium	Colpidia Introduced	Increase during Successive 5-Day Periods per Individual Introduced						
		First	Second	Third	Fourth	Fifth	Sixth	Seventh
dl-alanine	2,000	39.5	1.9	13.0	dead	.....	.....	.....
	2,000	23.8	13.5	7.8	dead	.....	.....	.....
d-arginine monohydrochloride	2,000	12.9	8.8	8.8	9.5	3.7	3.3	dead
	2,000	39.3	8.2	6.2	7.5	8.6	3.7	dead
dl-aspartic acid	2,000	3.6	1.1	dead	.....	.....	.....	.....
	2,000	2.3	2.6	dead	.....	.....	.....	.....
l-cystine 0.00045 M	2,000	10.8	10.1	8.3	2.7	dead	.....	.....
	2,000	9.5	7.9	8.2	8.2	7.9	dead	
dl-glutamic acid monohydrate	2,000	6.8	2.4	dead	.....	.....	.....	.....
	2,000	5.7	3.5	dead	.....	.....	.....	.....
Glycine	2,000	19.5	5.9	17.8	15.9	0.3	1.5	dead
	2,000	18.0	6.7	19.6	5.4	1.8	4.7	dead
l-histidine monohydrochloride	2,000	4.9	1.0	1.0	dead	.....	.....	.....
	2,000	3.9	1.0	1.0	dead	.....	.....	.....
l-hydroxyproline	2,000	40.7	1.6	7.5	5.2	5.8	dead	.....
	2,000	32.8	1.7	10.6	3.5	6.0	dead	.....
dl-isoleucine	2,000	11.7	2.1	dead	.....	.....	.....	.....
	2,000	10.7	1.9	dead	.....	.....	.....	.....
dl-leucine	2,000	11.5	2.7	dead	.....	.....	.....	.....
	2,000	10.7	3.3	dead	.....	.....	.....	.....
dl-lysine monohydrochloride	2,000	19.4	2.4	2.7	2.8	dead	.....	.....
	2,000	15.6	2.3	1.7	dead	.....	.....	.....
dl-methionine	2,000	12.7	2.5	dead	.....	.....	.....	.....
	2,000	14.6	2.8	dead	.....	.....	.....	.....
dl-phenylalanine	2,000	22.6	0.7	dead	.....	.....	.....	.....
	2,000	23.3	0.6	dead	.....	.....	.....	.....
l-proline	2,000	26.4	1.6	22.0	dead	.....	.....	.....
	2,000	24.7	1.6	9.9	dead	.....	.....	.....
dl-serine	2,000	41.5	3.4	4.6	18.7	0.9	4.4	dead
	2,000	38.5	3.6	6.6	6.7	4.2	4.4	dead
dl-threonine	2,000	10.0	3.1	9.2	4.1	9.6	1.3	dead
	2,000	8.1	6.2	10.1	4.6	6.8	7.1	dead
l-tryptophane	2,000	6.7	1.4	dead	.....	.....	.....	.....
	2,000	6.1	1.0	dead	.....	.....	.....	.....

TABLE 6—*Continued*

Amino Acid Present in Medium	Colpidia Introduced	Increase during Successive 5-Day Periods per Individual Introduced						
		First	Second	Third	Fourth	Fifth	Sixth	Seventh
l-tyrosine 0.000265 M	2,000	6.4	0.6	dead	.....	.....	.....	.....
	2,000	6.4	0.5	dead	.....	.....	.....	.....
dl-valine	2,000	9.7	0.5	8.9	dead	.....	.....	.....
	2,000	8.7	0.3	dead	.....	.....	.....	.....
No amino acid	2,000	dead	.....	.....	.....	.....	.....	.....
	2,000	dead	.....	.....	.....	.....	.....	.....
Tryptone	2,000	9.8	11.0	9.9	13.9	17.5	13.2	10.6
	2,000	18.1	10.4	14.4	15.9	9.7	17.0	18.6

and histidine, respectively; 25 days in that containing hydroxyproline; and 30 days in those containing arginine, glycine, serine, and threonine, respectively; but that in the medium with no amino acids they did not live 5 days and in that containing tryptone they were still reproducing normally at the close of the experiment.

These results indicate that no single amino acid will indefinitely support growth and reproduction in colpidia but that in media containing arginine, glycine, serine, threonine, or hydroxyproline, respectively, they live surprisingly long and reproduce initially at a comparatively high rate. The fact that they live so long in media containing these single amino acids seems to show that they can synthesize various other amino acids from them; and it indicates that it may be possible, by adding various other substances, to extend indefinitely the length of life in media which contain but one amino acid.

DISCUSSION

Rose (1938) and his co-workers found that rats required ten amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine. The results presented in the preceding pages show that colpidia will not grow on these but that they will grow if alanine, aspartic acid, cystine, glutamic acid, glycine, hydroxyproline, proline, serine, and tyrosine are added, and that, of the ten amino acids required by rats, colpidia do not need histidine, leucine, lysine, or threonine. Colpidia consequently require some amino acids which rats do not need, and vice versa; but they require five more acids than rats. Hence, if all nineteen amino acids are present in the proteins of colpidia and rats, it is evident that colpidia cannot synthesize missing amino acids to the same extent as rats and that their ability to synthesize differs also in kind from that possessed by rats. It has been demonstrated that the proteins in rats contain these amino acids, but nothing is known concerning the number and kind of amino acids in the proteins in colpidia.

Mast and Pace (1933) found, contrary to the contention of Pringsheim (1921), that *Ch. paramecium* can synthesize proteins from inorganic salts. Its power of synthesis is therefore obviously much greater than either that of rats or that of colpidia.

The results presented above strongly indicate that colpidia can grow indefi-

nitely on amino acids if the proper salts and vitamins are present. What these vitamins are is a subject for further research.

Hall and Elliott (1938) failed, as stated above, to obtain growth of colpidia on the incomplete proteins, gelatin, and zein, even when they supplemented them with the missing amino acids; but they did obtain growth on complex protein derivatives, such as are found in Bacto-peptone and Proteose-peptone, i.e., crude protein hydrolysates sold under these tradenames.

Gelatin and zein are usually much more thoroughly purified than the protein derivatives in the Bacto products and consequently probably contain fewer vitamins. It may well be, then, that the failure to obtain growth in colpidia on gelatin or zein with the missing amino acids added was due to a lack of vitamins rather than to an insufficiency of appropriate nitrogen compounds.[5]

Elliott (1935) attempted to culture colpidia in five media each of which contained only one amino acid, namely, glycine, leucine, phenylalanine, valine, or asparagin. (There is considerable doubt that asparagin is an amino acid; it would probably be better to consider it an amino acid amide.) He obtained growth in all, but only for a few days; and he concluded that this was due to the slight amount of tryptone carried over with the colpidia when they were introduced into the media and that no single amino acid suffices for growth in colpidia.

The results presented above show that colpidia can grow and reproduce for nearly 30 days in media which contain only one amino acid, for with the methods used the amounts of amino acids and other more complex protein derivatives carried over in transfer from the culture in which the colpidia were raised were, in all probability, negligible, and this was greatly reduced in each of the following transfers. The difference between these results and those obtained by Elliott was doubtless largely, if not entirely, due to differences in the vitamins present. Moreover, there is some evidence in hand which indicates that, by the addition of other vitamin complexes than those used, the nutritional requirements of colpidia can be further simplified.

The connection between the presence of vitamins and the synthesis of amino acids here indicated is one of the most interesting implications of this investigation, and it is hoped that further researches concerning it will be forthcoming soon.

[5] In a paper published since this work was done, Hall (1942) supports this tentative conclusion.

SUMMARY

1. A medium consisting of fifteen of the twenty-one amino acids present in casein, eight vitamins, inorganic salts, dextrose, and water supports growth in *C. striatum* indefinitely. Growth will not continue indefinitely if any one of the fifteen amino acids is omitted; all are necessary.

2. The ten amino acids necessary for growth in rats do not suffice for growth in *C. striatum*.

3. If the culture medium contains only one of nineteen of the twenty-one amino acids present in casein, the colpidia will not grow and reproduce indefinitely. However, if the culture medium contains arginine, glycine, serine, or threonine, respectively, the colpidia grow and reproduce for a month, possibly indefinitely if the proper vitamins are present.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

BIOLOGICAL SYMPOSIA, VOLUME IX: *SEX HORMONES*[1] EDITED BY F. C. KOCH AND PHILIP E. SMITH

Reviewed by EMIL WITSCHI
University of Iowa

THE ninth volume of the *Biological Symposia* contains two series of lectures on *Sex Hormones* by eight renowned research workers. One series was presented at the Fiftieth Anniversary Celebration of the University of Chicago in September, 1941; the other at the annual meeting of the American Association of Anatomists at New York City in April, 1942.

In the short Foreword to the Chicago symposium on "The Comparative Biology and Metabolism of the Testicular and Ovarian Hormones," Frank R. Lillie fittingly comments on the immense impetus which research in biology of sex received from the application of chemical methods. C. R. Moore discusses some of the relativities and complexities in present-day concepts of sex, whether based primarily on hormonal, morphological, genetical, or behavioristic data. Indicating many problems in obvious need of further elucidation, he also emphasizes that the direction of future progress will depend largely on the discovery of entirely new facts. F. C. Koch and E. A. Doisy survey and contribute new mate-

[1] Lancaster, Pa.: Jacques Cattell Press, 1942. Pp. x+146+47 figs. $2.50.

rials to the knowledge concerning the role of the sex hormones in the metabolism of healthy and diseased organisms. While the sex glands clearly are the sites where the bulk of male and female sex hormones are synthesized, the chemical detail of the processes of manufacture is still largely unknown. Considerable progress, however, is reported with respect to the chemical identification of the hormones, their distribution in the organism, and the transformations which they suffer in the liver and the kidneys. A. T. Kenyon extends this presentation by a description of the effects of experimentally administered sex hormones on growth and general metabolism, as observed in clinical investigations.

The second symposium is concerned with the more specialized subject of "Hormonal Factors in the Inversion of Sex." Strictly speaking, only R. R. Humphrey deals with sex inversion, in his excellent review of the work on amphibians. The other three papers give consideration almost exclusively to the control of secondary sex characters. C. H. Danforth, in a discussion of sexual dimorphism in bird plumages, stresses the collaboration of genetical (chromosomal) and hormonal factors in the determination of the patterns of color and structure of feathers. R. K. Burns, reviewing the important experiments on pouch opossums, likewise finds the possibilities of hormonal control limited by the genetical (male or female) constitution of the reacting tissues. While crystalline sex hormones often elicit truly astounding reactions, Burns does not believe that this necessarily constitutes proof that such substances are actually involved in the normal differentiation of sex. R. R. Greene, however, reporting on the effects of sex hormones on embryonic sexual structures of the rat, favors, at least "as an interesting speculation," the surmise that the embryonic gonads produce sex hormones in considerable quantity and by this means fully control the sexual differentiation of the genital ducts, the external genitalia, and the mammary apparatus. It is interesting to compare Greene's conclusions with a statement by C. R. Moore, in the same volume, to the effect that the earliest dependable evidence for sex hormone secretion "comes from somatic changes indicative of approaching sexual maturity."

It is fortunate that this volume offers a variety not only of facts but also of interpretations. The presentation of conclusions that reveal a degree of divergence in the theoretical evaluation of the vast body of experimental data, even among the best-informed investigators, will, no doubt, stimulate new investigations, which may become crucial for the solution of now controversial issues.

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